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<b>(54) Title:</b> GENE THERAPY USING TARGETED VIRAL VECTORS  <b>(57) Abstract</b>  A general method for delivering genes to specific target cells <i>in vivo</i> is described. Enveloped viruses are genetically engineered to infect specific target cells by replacing the cell surface receptor recognition domain of viral envelope proteins with ligands that direct the binding and fusion of these viruses to specific cell surface molecules.		

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GENE THERAPY USING TARGETED VIRAL VECTORSBackground of the Invention

The invention relates to gene therapy methods.

5 Gene therapy is an approach to treating a broad range of diseases by delivering therapeutic genes directly into the human body. Diseases that can potentially be cured by gene therapy include 1) diseases associated with the aging population such as cancer, 10 heart disease, Alzheimer's disease, high blood pressure, atherosclerosis and arthritis; 2) viral infectious diseases such as acquired immune deficiency syndrome (AIDS) and herpes; and 3) inherited diseases such as diabetes, hemophilia, cystic fibrosis, and muscular 15 dystrophy.

Current methods of delivery of new genetic information into cells *in vitro* include cell fusion, chromosome-mediated insertion, microcell-mediated gene transfer, liposome DNA carriers, spheroplast fusion, DNA- 20 mediated gene transfer, microinjection, infection with recombinant RNA viruses, and infection with recombinant DNA viruses (Martin, J.C., 1984, *Mol. Cell Biochem.* 59:3-10). These techniques are not generally applicable, however, for use in animals or humans because of low 25 efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and lack of target specificity.

In one particular example, a favored approach for human gene therapy involves the transplantation of 30 genetically-altered cells into patients (Rosenberg, et al., 1988, *New Eng J Medicine* 323:570-578). This approach requires the surgical removal of cells from each patient to isolate target cells from nontarget cells. Genes are introduced into these cells via viral vectors 35 or other means, followed by transplantation of the

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genetically-altered cells back into the patient. .  
Although this approach is useful for purposes such as  
enzyme replacement therapy (for example, for  
transplantation into a patient of cells that secrete a  
5 hormone that diseased cells can no longer secrete),  
transplantation strategies are less likely to be suitable  
for treating diseases such as cystic fibrosis or cancer,  
where the diseased cells themselves must be corrected.  
Other problems commonly encountered with this approach  
10 include technical problems, including inefficient  
transduction of stem cells, low expression of the  
transgene, and growth of cells in tissue culture which  
may select for cells that are predisposed to cancer.  
Finally, inappropriate expression of transplanted genes  
15 in nontarget cells may actually be harmful to patients.

An alternative approach to gene therapy involves  
the direct delivery of genes to target tissue *in situ*.  
Two methods for *in situ* delivery of genes have been  
developed: biolistic transfer and double balloon  
20 catheterization. Biolistic transfer of genes involves  
shooting DNA-coated platinum or gold microprojectiles  
directly into target tissue. Biolistic transfer has been  
successful in the transient expression of genes in the  
ear, skin and surgically-exposed liver of live mice  
25 (Johnston, S.A., 1990, *Nature* 346:776-777; Williams,  
R.S., et al., 1991, *Proc Natl Acad Sci USA* 88:2726-2730).  
Double balloon catheterization transduces genes into  
cells within a defined arterial wall segment. In this  
approach a double balloon catheter is inserted into an  
30 artery until the end of the catheter is located within  
the target area. Inflation of two balloons at the end of  
the catheter creates an enclosed space into which  
retrovirus or DNA-loaded liposomes are infused. This  
method has been successful in the transient expression of  
35  $\beta$ -galactosidase genes within a defined segment of the

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ileofemoral artery of pigs (Nabel E.G., et al., 1990, *Science* 249:1285-1288). Both biolistic transfer and double balloon catheterization however, although locally specific, may be nonspecific in the individual cells that they transduce within the target area, creating a problem of inappropriate gene regulation if the transgene is expressed in nontarget cells. Moreover, neither biolistic transfer nor double balloon catheterization have been shown to be effective for the treatment of tissue occupying large volumes such as lungs, muscles, tumors, or cells of the systemic circulation since the majority of the cells would be inaccessible for *in situ* gene transfer.

A third approach to gene therapy is the delivery of genes to cells *in vivo*. This approach involves the introduction of viral vectors directly into patients by injection, spray or other means. Different species of viruses are engineered to deliver genes to the cells that the viruses normally infect. Adenovirus, for example, which normally infects lung cells, has been developed as a vector to target genes to lung cells (Rosenfield, et al., 1992, *Cell* 68 143-155). Most viral vectors, however, are single purpose vectors since they can only deliver genes to certain cells. Because the target cell specificity of viral vectors is restricted to the normal tropisms of the viruses, viral vectors are generally limited in that they either infect too broad a range of cell types, or they do not infect certain types of cells at all.

Liposomes have been designed to deliver genes or drugs to specific target cells *in vivo*. By chemically conjugating antibodies or ligands to liposomes, liposomes have been targeted to specific cells. With this method, antisense *env* RNA has been delivered to human immunodeficiency virus (HIV)-infected lymphocytes using

anti-CD3-conjugated liposomes (Renneisen, K., et al., 1990, *J Biol Chem* 265:16337-16342); chloramphenicol transacetylase (CAT) genes have been delivered to H2K<sup>k</sup> positive lymphomas in H2K<sup>k</sup>-negative nude mice using anti-H2K<sup>k</sup>-conjugated liposomes (Wang, C. et al., 1987, *Proc Natl Acad Sci USA* 84:7851-7855); and xanthine guanine phosphoribosyltransferase (XGPRT) genes have been delivered to immunoglobulin-coated cells using staphylococcus protein A-conjugated liposomes (Machy P., et al., 1988, *Proc Natl Acad Sci USA* 85:8027-8031). The major drawback to this technology can be the expense of mass producing ligand-conjugated liposomes.

Wu et al. report a method to target naked DNA to specific cells. Asialoglycoprotein-DNA complexes are targeted to hepatocytes expressing the asialoglycoprotein receptor (Wu G.Y., et al., 1991, *Biotherapy* 3:87-95). Similar to the problem encountered with immunotoxins, however, this strategy generally limits delivery of DNA to cells expressing receptors that are capable of DNA-internalization.

Antisense DNA technology is a method for inhibiting the expression of specific genes with complementary DNA (Moffat, 1991, *Science* 253:510-511). Although antisense DNA is specific in the genes that it affects, it is nonspecific in the types of cells that it gets into. This can create problems *in vivo* because it is desirable that endogenous genes in normal cells remain unaffected by antisense DNA (e.g., protooncogenes). Moreover, the cost of manufacturing and administering antisense DNA may be high because the phosphate moieties of antisense DNA must be chemically modified to allow passage through the plasma membrane, a process which entails expensive organic chemistry. Millimolar concentrations of antisense DNA are required to be effective, posing problems of potential toxicity *in vivo*.

Human gene therapy is therefore limited by the available technology for gene delivery. Transplantation strategies, which require surgery, limit gene therapy to an expensive service industry for a small number of diseases. Targeting of genes *in situ* through local transduction is generally not precise enough. Viral vectors limit the delivery of genes *in vivo* to cells that the viruses normally infect. Liposome technologies may be infeasible because of the expense of production. Simple ligand-DNA complexes will not introduce genes into cells unless the receptors, against which the ligands are directed, internalize. Accordingly, currently available gene delivery systems impose severe limitations on the spectrum of diseases that can be treated by gene therapy.

#### Summary of the Invention

The invention features a method for expressing a nucleic acid of interest in a heterologous host cell. The method involves providing a virus whose genome comprises i) the nucleic acid of interest, and ii) a hybrid envelope gene. The hybrid gene encodes an envelope fragment joined to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding of its normal host cell but does facilitate efficient incorporation of the virus into a mature viral particle, and whereby the targeting ligand facilitates targeting and binding of the mature viral particle to the surface of the heterologous host cell. The method also involves administering the virus so as to permit viral infection of the cell.

By "efficient incorporation", is meant that the hybrid envelope protein is incorporated into a mature viral particle at least 25% as frequently as the corresponding wild-type envelope protein is incorporated into a mature viral particle.

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In another aspect the invention features a virus, the genome of which encodes a hybrid envelope protein, wherein the hybrid protein comprises an envelope fragment joined in frame to a targeting ligand, whereby the  
5 envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of the hybrid envelope protein into a mature viral particle and whereby the non-viral protein facilitates targeting and binding of the mature  
10 viral particle to the surface of a cell not normally infected by the virus.

In a third aspect the invention features a method for delivering a nucleic acid of interest to a heterologous host cell. The method involves providing a  
15 virus that comprises i) the nucleic acid of interest, and ii) a hybrid envelope gene, the hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding to its normal host cell but does facilitate  
20 efficient incorporation of the virus into a mature viral particle, and whereby the targeting ligand facilitates targeting and binding of the mature viral particle to the surface of the heterologous host cell. The method also involves administering the virus so as to permit viral  
25 infection of the cell.

In various preferred embodiments the virus is an enveloped virus, preferably a Herpesviridae, a Paramyxoviridae, or a Retroviridae, most preferably a Moloney murine leukemia virus, or the virus may also  
30 preferably be a Hepadnaviridae, a Poxviridae, or an Iridoviridae. Similarly the virus may be a Togaviridae, a Flaviviridae, a Coronaviridae, a Rhabdoviridae, a Filoviridae, an Orthomyxoviridae, a Bunyaviridae, or an Arenaviridae, or any other, yet unclassified, enveloped  
35 virus.



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In other various preferred embodiments the nucleic acid of interest may include, without limitation, an antisense oncogene; a tumor suppressor gene, e.g., a gene encoding p53, or a gene encoding retinoblastoma protein Rb; a toxin gene, e.g., a diphtheria toxin gene; or a gene encoding a cytokine, e.g., a tumor necrosis factor, or an interferon. The nucleic acid of interest may be either DNA or RNA, e.g., antisense DNA, or antisense RNA, or a nucleic acid encoding an antisense RNA. The nucleic acid of interest may also be a gene invoking intracellular immunity, or a nucleic acid therapeutic for an inherited disease, e.g., an insulin gene, or a cystic fibrosis transmembrane regulator gene. A "gene that invokes intracellular immunity" is a gene that confers a dominant negative resistant phenotype to the cell it is in, thereby protecting the cell against an invading agent.

The heterologous host cell may be a cell that has acquired mutations that result in a disease state, preferably a cancer cell, e.g., a colon cancer cell. The heterologous host cell may be a cell infected with a second virus, e.g., a human immunodeficiency virus (HIV), a cell infected with an organism, or an infectious agent such as a bacterium or parasite. The infectious agent may be either unicellular or multicellular. The heterologous host cell may also be a cell affected by a hereditary disease, e.g., a pancreatic beta cell, or a lung cell.

The targeting ligand, in additional various preferred embodiments, may include a protein, preferably a hormone, or an immunoglobulin, more preferably an anti-tumor associated antigen-specific immunoglobulin, most preferably an anti-carcinoembryonic antigen-specific immunoglobulin, or an anti-HIVgp120 antigen-specific immunoglobulin. The targeting ligand may also be a

carbohydrate, or a lipid. The hybrid envelope fragment may consist of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, a signal sequence, and preferably a fusion domain. In some cases the fusion activity of the envelope fragment may be performed by a second protein. The second protein would therefore direct fusion of the virus with the membrane of the targeted cell.

10 The mode of administration may include, but is not limited to, 1) direct injection of the purified virus; or 2) implanting a container enclosing the virus into a patient. When the virus is administered inside a container, the virus is preferably inside a packaging cell. A "packaging cell" is a cell that supplies viral proteins necessary for production of viral vectors. By "container" is meant a virus permeable enclosure containing virus, or containing packaging cells with virus therein.

20 "Normal host cell" as used herein, is a cell type commonly infected by the naturally occurring virus. In contrast, the term "heterologous host cell" or a "targeted cell", as used herein, refers to a cell that is recognized as a function of the targeting ligand portion of the hybrid envelope protein, but is not recognized as a function of the envelope portion of the hybrid envelope protein. By "targeting ligand" is meant a molecule that has binding affinity for a molecule on the surface of a desired targeted cell. A "hybrid envelope protein", as used herein, is a protein that includes a portion of a viral envelope protein (or a biologically active analog thereof) covalently linked to a targeting ligand. For example, by a "hybrid immunoglobulin-env protein" is meant a portion of an immunoglobulin covalently linked to 35 a portion of an envelope protein. A "hybrid envelope

gene" is a nucleic acid that provides genetic instructions for a hybrid envelope protein. By "hybrid anti-carcinoembryonic antigen-specific immunoglobulin" is meant a hybrid immunoglobulin-env protein that  
5 specifically binds to a carcinoembryonic antigen.

The term "fragment", as applied to an envelope protein fragment, includes some but not all of the envelope protein. A fragment will ordinarily be at least about about 20 amino acids, typically at least about 30  
10 amino acids, usually at least about 40 contiguous amino acids, preferably at least about 50 amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Fragments of an envelope protein can be generated by methods known to those skilled in the  
15 art (e.g., those described herein).

A biologically active fragment of a viral envelope protein is one that possesses at least one of the following activities: a) it can bind to a cell membrane if given the appropriate targeting ligand; b) it can  
20 enable fusion with a cell membrane; or c) it can enable incorporation of proteins into a mature viral particle. These three biological activities can be performed by the same envelope protein fragment, or by two separate envelope protein fragments. As stated above, the  
25 envelope fragments of this invention do not facilitate recognition or binding of the virus' normal host cell. This is accomplished by either destroying the activity of the normal receptor binding region by mutation, or by physically deleting it. A new recombinant receptor-  
30 binding region is added in its place. The ability of a candidate fragment to exhibit a biological activity of a viral envelope protein can be assessed by methods known to those skilled in the art.

The envelope fragment may include the amino acid  
35 sequence of a naturally-occurring viral envelope or may be

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a biologically-active analog thereof. The biological activity of an envelope analog is assessed using the methods described herein for testing envelope fragments for activity.

- 5 Applicants have provided an efficient and reliable means for specifically delivering therapeutic genes or antisense nucleic acids to particular animal, plant or human cell types, or to cells of infectious agents. Their method facilitates treatments for mutagenically  
10 acquired, infectious, or inherited diseases, e.g., by either 1) antagonizing the effect of an existing cellular gene; 2) complementing the defect of an existing cellular gene; 3) destroying the target cells through the introduction of new genetic material; or 4) changing the  
15 phenotype of the target cells through the introduction of new genetic material. To specifically target cells for delivery, a hybrid envelope protein (e.g., an envelope-antibody or envelope-ligand hybrid) is utilized which directs specific interaction with a particular target  
20 host cell. The viruses itself, through its efficient internalization mechanisms, facilitates efficient uptake of the therapeutic gene. Such viral vectors are uniquely adapted to deliver genes, RNA, or drugs to cell surface proteins that do not normally internalize.
- 25 Another advantage of this invention is that it overcomes the problem of gene regulation encountered with other methods of gene therapy. Genetically-altered cells must not only synthesize the gene products at the right location, at the right time, and in the right amounts,  
30 but must also be regulated in the same manner as the indigenous tissue. That is, the transduced cells must also have all the proper signal transduction mechanisms to respond to extracellular signals. This may be a  
35 transplanting fibroblasts with insulin genes can be

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ineffective or even harmful. As fibroblasts do not contain the same receptors and signal transduction machinery as pancreatic beta cells, the insulin genes may be expressed differently. Targeting genes to the right  
5 cells insures that the genes will be properly regulated.

In an additional aspect of the invention, a selection scheme is devised for creation of hybrid envelope protein-containing viruses. This strategy will be feasible for env proteins fused with immunoglobulins  
10 or with any ligand that recognizes specific receptors on cells.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

15

#### Detailed Description

The drawings will first briefly be described.

#### Drawings

FIG. 1 is a representation of a scheme for constructing retroviral vector pLNCX\*.

20 FIG. 2 is a representation of a scheme for constructing plasmid LNCenvpA.

FIG. 3 is a representation of a scheme for constructing plasmid LNCenv.

25 FIG. 4 is a representation of a scheme for constructing plasmid pUC Star-Sig.

FIG. 5 is a representation of a scheme for constructing plasmid LNC-Sig.

FIG. 6 is a representation of a scheme for constructing plasmid LNC-antiCEA.

30 FIG. 7 is a representation of plasmids used in the construction of targeted viruses.

FIG. 8 is a representation of a strategy for generating targeted retroviruses involving construction of hybrid immunoglobulin-env genes *in vitro*.

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FIG. 9 is a representation of a strategy for generating targeted retroviruses involving generation of pooled virus constructions.

FIG. 10 is a representation of a strategy for generating targeted retroviruses involving selection and characterization of targeted virus.

FIG. 11 is a representation of a plasmid containing a targeted retroviral vector.

FIG. 12 is a representation of a scheme for constructing plasmid pUC Star-antiCEA.

FIG. 13 is a representation of an alternative scheme for constructing plasmid LNC-antiCEA.

What follows is a procedure for the delivery of genes to target cells using targeted viral vectors. To create and target a virus, the receptor recognition domain of the viral envelope protein is replaced with a ligand directed against a specific cell surface receptor. The hybrid envelope protein is incorporated into the viral envelope during the budding process, producing a hybrid virus *in vivo*. Upon infection of a host, the hybrid virus specifically recognizes its target cell and resultant fusion with that cell facilitates internalization (into the target cell) of viral genes, including the therapeutic gene(s) which are engineered into the viral genome. Such internalization can be extremely important; for example, immunotoxins, although efficient at delivering toxin molecules to target cells, are often clinically ineffective since the cell surface molecules to which they are targeted do not internalize, and internalization is required for entry of the toxin molecules into the cells (Waldmann, T.A., 1991, *Science* 252: 1657-1662). Targeted viruses circumvent this requirement for receptor internalization since the virus itself contains the necessary cell fusion machinery (Gilbert, J.M., et al., 1990, *J Virol* 64: 5106-5113;

Roizman, B. et al., 1990, in BN Fields, et al., eds.  
Virology, Raven Press, Ltd. New York).

General Requirements for Targeted Viruses

In general targeted viruses are constructed by  
5 replacing the receptor recognition domain of the viral  
envelope protein with a ligand directed against a  
specific cell surface receptor. The ligand can be,  
without limitation, an immunoglobulin (e.g., FAb, dAb,  
Fd, or Fc), a hormone, or any other synthetic or natural  
10 protein that can direct the binding of the targeted  
viruses to a cell surface molecule. The ligand is  
biologically incorporated into the viral envelope by  
genetic fusion with that portion of the normal viral  
envelope protein involved in viral assembly and budding.  
15 The envelope portion of the hybrid protein consists of an  
envelope fragment (or analog thereof) that is sufficient  
to direct efficient incorporation of the envelope hybrid  
protein into the viral envelope. Preferably, the  
envelope hybrid protein no longer directs an interaction  
20 between the virus and its normal host cell.

It has been demonstrated that changing the  
receptor specificity of the envelope protein of a virus  
changes the virus's tropism. For example, vesicular  
stomatitis virus (VSV) pseudotypes that have their virus  
25 envelope replaced with that of a retrovirus acquire the  
ability to infect retrovirus infectable cells (Schnitzer,  
T.J., et al., 1977, *J Gen Virol* 23:449-454; Zavada. J.,  
et al., 1972, *J Gen Virol* 15:183-191), indicating that  
species specific protein-protein interactions between a  
30 virus core protein and an envelope protein are not  
critical for virus fusion and penetration at least in  
these cases. Moreover, previous experiments have also  
indicated that virus envelopes can tolerate changes in  
length or conformation introduced into the envelope  
35 protein, e.g., by a conjugated ligand. For example,

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Gitman et al. have shown that Sendai virus envelopes reconstituted with viral envelope glycoproteins, chemically cross-linked to anti-erythrocyte antibodies acquire the ability to bind to erythrocytes that had been  
5 stripped of the normal virus receptor. Similarly, Sendai virus envelopes reconstituted with envelope proteins, chemically cross-linked to insulin molecules, were able to bind to receptor-stripped erythrocytes expressing the insulin receptor. In both cases, envelope binding but  
10 not fusion occurred with the receptor-stripped erythrocytes. Fusion between the conjugated envelopes and erythrocytes occurred, however, when the conjugated-envelopes were coreconstituted with the normal viral hemagglutinin/neuraminidase and fusion proteins (Gitman,  
15 A.G., et al., 1985, *Biochem* 24:2762-2768).

Preferably, the targeted virus contains 1) a viral envelope derived from a host cellular membrane; 2) a transmembrane hybrid envelope protein that directs the binding and penetration of the virus to specific target  
20 cells; 3) a transmembrane envelope protein that directs the fusion of the targeted virus with the cellular membrane of the targeted cell for viral penetration (e.g., the targeting protein itself or another envelope protein); 4) viral core proteins; 5) a foreign gene(s) of  
25 interest; and 6) all necessary viral and genetic components for penetration and expression of genes contained in the viral genome. The transmembrane hybrid envelope protein consists of 1) determinants that enable the hybrid protein to become processed and incorporated  
30 into viral envelopes; 2) determinants that enable fusion of the viral envelope with the targeted cellular membrane; these are essential for penetration of the targeted virus; 3) a ligand determinant that enables the targeted virus to recognize and bind to specific  
35 receptors on target cells. The viral genome may also



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include bacterial selectable markers (e.g., ampicillin resistance) and/or a mammalian cell selectable marker (e.g., neomycin resistance).

The transmembrane hybrid protein is constructed  
5 genetically by splicing the cell surface receptor binding domain of a ligand gene to a portion of the viral envelope protein gene. The transmembrane hybrid protein must retain those portions of the envelope protein that direct the efficient post-translational processing,  
10 sorting and incorporation of the protein into the viral envelope.

The following domains must be considered in constructing the hybrid ligand-envelope protein:

1. The receptor binding domain

15 The receptor binding domain is that portion of the envelope protein that recognizes and binds to cell surface receptors. In hybrid envelope proteins, this portion of the envelope protein is replaced with ligand sequences. The receptor binding domain of retrovirus  
20 envelope proteins has been localized to the SU subunit (Coffin, J.M., 1990, in BN Fields, et al., eds. Virology, Raven Press, Ltd., New York). Since the SU protein of retroviruses is coded for 5' to the transmembrane protein, replacement of the amino-terminal sequences of  
25 the envelope protein with ligand sequences poses no problem for the creation of a functional hybrid ligand-envelope protein.

2. Proteolytic cleavage site

The envelope protein of retroviruses is  
30 synthesized as a polyprotein which is later proteolytically cleaved to form SU and TM heterodimers. In construction of the hybrid ligand-envelope protein, the proteolytic cleavage site should be eliminated. The proteolytic cleavage site should be eliminated either by  
35 deletion or by site directed mutagenesis. Perez and

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Hunter have demonstrated that elimination of the proteolytic cleavage site does not block transport or surface expression of Rous sarcoma virus envelope proteins (Perez, L.G., et al., 1987, *J Virol* 61:1609-1614).

### 3. Oligomerization domain

The envelope proteins of many animal viruses associate to form trimers (Fields, B.N., et al., 1991, *Virology*, 2nd ed., Raven Press, Ltd., New York). Trimerization of the envelope protein is thought to be essential for the proper transport and insertion of envelope proteins into the viral envelope (Singh, J. et al., 1990, *Embo J* 9:631-639; Kreis, T.E., et al., 1986, *Cell* 46:929-937). Therefore it is important that this domain be retained in the hybrid ligand-envelope protein. The trimerization domain likely resides in the transmembrane TM protein of retroviruses (Einfeld, D., et al., 1988, *Proc Natl Acad Sci USA* 85:8688-8692); hence, creation of a functional hybrid ligand-envelope retroviral protein lacking the SU subunit is possible. Some viral envelope proteins may oligomerize to form stoichiometric combinations other than trimers.

### 4. Fusion domain

The fusion domain is a hydrophobic stretch of amino acids that is involved in fusion of the virus envelope with the cell membrane (Wiley, D.C., et al., 1990, in Fields, B.N. et al., eds. *Virology*, 2nd ed., Raven Press, Ltd., New York). Viral fusion allows entry of the viral core proteins and genome into the cell. In influenza virus, the fusion domain, located in the amino terminus of the envelope HA2 protein, is sequestered in the hemagglutinin trimer until a low pH-induced conformational change allows presentation of the fusion domain to the cell membrane. Trimerization of the envelope proteins can prevent constitutive expression of

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fusion activity by sequestering it within an internal hydrophobic pocket. A potential fusion domain has been located within the extracellular portion of the gp37 TM protein of Rous sarcoma virus (Hunter E. et al., 1983, *J Virol* 46:920-936). Similar hydrophobic fusion sequences have been noted in the p15E protein of Moloney murine leukemia virus (Mo-MuLV) (Chambers, P., et al., 1990, *J Gen Virol* 71:3075-3080).

In constructing a hybrid ligand-envelope protein, it may be necessary to eliminate the fusion domain to prevent the possibility of constitutive fusion activity, a state that may impair the infectivity of targeted viruses. Therefore two proteins may be incorporated into the viral envelope of targeting viruses. The first protein is the hybrid ligand-envelope protein which directs targeting of the virus but lacks fusion activity. The second protein is an envelope protein possessing fusion activity but lacking a receptor binding domain. This type of situation is observed for paramyxoviruses where one envelope protein is dedicated to targeting while another carries out fusion (Kingsbury, D.W., 1990, B.N. Fields, et al., eds. *Virology*, 2nd ed., Raven Press, Ltd., New York.). Where it is not necessary to prevent constitutive fusion activity, both activities may be included in one protein.

#### 5. Transmembrane domain

The transmembrane domain is a stretch of approximately twenty or more amino acids that anchor the envelope protein to the viral envelope. It is located within the p15E protein of Moloney murine leukemia virus (Chambers, et al., *supra*). Retention of the transmembrane domain is thought to be essential since deletion of the transmembrane domain results in secretion of the synthesized envelope protein (Perez, L.G., et al., 1987, *J Virol* 61:2981-2988).

#### 6. Virus budding domain

Amino acid sequences within the envelope protein may be involved with the exclusive incorporation of viral envelope proteins into viral envelopes and with virus budding. The virus budding domain directs the hybrid ligand-envelope protein into the viral envelope. These sequences are thought to reside within the portion of the envelope protein facing the inside of the virus and may involve specific protein-protein interactions between envelope proteins and viral core or matrix proteins. Although Perez et al. demonstrated that deletion of the carboxy-terminal sequences of the Rous sarcoma virus env protein resulted in normal budding of the mutant virus (Perez, L.G., et al., 1987, *J Virol* 61:2981-2988), evidence exists that, for other viruses, interactions between envelope proteins and viral core proteins may direct virus assembly and envelopment (BN Fields, et al., eds., 1990, *Virology*, Raven Press, Ltd., New York).

#### 7. Sorting signals and other signals

Sorting signals are determinants that direct the envelope protein to the correct intracellular location during post-translational processing. These sequences insure that the envelope protein passes through the endoplasmic reticulum, Golgi apparatus, and other organelles until it eventually reaches the viral envelope. Other signals that may have to be retained in the hybrid ligand-envelope protein are glycosylation sequences and sequences involved in effective conformation of the envelope protein (e.g., disulfide bonds).

#### 8. Signal sequence

The signal sequence is an amino-terminal hydrophobic stretch of amino acids that directs the envelope protein into the endoplasmic reticulum. The signal sequence, which is later proteolytically cleaved,

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is essential for the hybrid ligand-envelope protein to become located in a membrane.

The diversity of signals and domains that must be considered in constructing targeted viruses requires that precise and correct splicing of ligand and envelope genes occur. The present invention describes a selection scheme for constructing targeted viruses whereby the ligand gene is spliced to an envelope gene fragment; this hybrid gene codes for those portions of the envelope protein which are required to direct efficient incorporation of the resultant hybrid envelope-ligand protein into the mature viral particle. According to the selection scheme, cell surface receptor binding domains of ligand genes are randomly ligated to progressive deletions of viral envelope genes. The correct combination of ligand and envelope sequences is determined by a selection scheme for the production of biologically active targeted virus. The selection scheme not only produces targeted virus but simplifies the construction of future targeted viruses.

#### A Specific Example of a Targeted Retrovirus

There now follows an example of a recombinant retrovirus which targets and infects particular host cells for the purpose of delivering to those cells a desired therapeutic gene. This example is provided for the purpose of illustrating, not limiting, the invention.

Moloney murine leukemia virus (Mo-MuLV) is a mouse ecotropic retrovirus. A recombinant Mo-MuLV based retroviral vector that is targeted to colon cancer cells is constructed. The targeted retroviral vector delivers the neomycin resistance gene to colon cancer cells. Targeting to human colon cancer cells is accomplished by incorporating into the viral envelope hybrid immunoglobulin-env proteins directed against carcinoembryonic antigen. Carcinoembryonic antigen (CEA)

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is a tumor associated antigen expressed on the surface of human colon cancer cells but not on the surface of normal adult cells. The CEA glycoprotein, possessing multiple membrane spanning alpha helices, does not internalize in response to ligand (Benchimol, S. et al., 1989, *Cell* 57:327-334). A protein that is homologous to carcinoembryonic antigen has recently been shown to be the receptor for mouse hepatitis virus (Dveksler, G.S., et al, 1991, *J Virol* 65:6881-6891).

For the purpose of this illustration, a single variable region of the heavy chain of anti-CEA is fused to a portion of the env gene. Single variable heavy chain fragments (dAb) have been shown to be as effective in antigen binding as fragmented antibodies (FAB), containing both heavy and light chain fragments, and intact monoclonal antibodies (Ward, E.S., et al., *Nature* 341:544-546). The function of immunoglobulin-env proteins is not limited, however, to the use of dAb's and can be applied with FAB's, Fv's and mAb's.

Modification of the retroviral vector LNCX

LNCX is a Moloney murine leukemia virus based retroviral vector contained in the plasmid pLNCX (Miller, A.D., et al., 1989, *Biotechniques* 7:980-990). pLNCX contains a unique HindIII and ClaI cloning site for expression of inserted genes, a cytomegalovirus (CMV) promoter, a polyadenylation site (pA), retroviral long terminal repeats (LTR) for retroviral RNA transcription and reverse transcription, a bacterial neomycin resistance gene (Neo) which conveys resistance to both neomycin and G418, a bacterial origin of replication (Or), a bacterial ampicillin resistance gene (Amp), and a retroviral RNA packaging sequence ( $\Psi$ +). LNCX is modified to contain a unique SalI site as shown in Figure 1. pLNCX is linearized with XbaI and subcloned into the XbaI site of the phagemid BluescriptII SK+ (Stratagene, La

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Jolla, CA). Single stranded DNA is purified and the unique BstEII site of LNCX is converted into a Sall site by site directed mutagenesis with the oligonucleotide 5'-GCAGAAGGTCGACCCAACG-3' (SEQ ID NO: 1). The BstEII site is located within the extended packaging signal ( $\Psi$ ) of Mo-MuLV RNA (Bender, M.A., et al., 1987, *J Virol* 61:1639-1646; Adam, M.A., et al., 1988, *J Virol* 62:3802-3806; Armentano, D. et al., 1987, *J Virol* 61:1647-1650). Conversion of the BstEII site to Sall does not affect packaging since this region has been determined to be dispensable for efficient packaging (Schwartzberg, P., et al., 1983, *J Virol* 46:538-546; Mann R. et al., 1985, *J Virol* 54:401-407; and Mann, R., et al., 1983, *Cell* 33:153-159). The BstEII site is converted into a Sall site because BstEII sites, but not Sall sites, frequently occur in heavy chain genes (Chaudhary, V.K., et al, 1990, *Proc Natl Acad Sci USA* 87:1066-1070). The Sall containing plasmid is recircularized with XbaI and DNA ligase to form the plasmid pLNCX\*.

20 Cloning of the Mo-MuLV env protein in pLNCX\*

The Mo-MuLV env gene is cloned into pLNCX\* as shown in Figure 2. The Mo-MuLV env gene is excised from plasmid p8.2 (Shoemaker, C., et al., 1980, *Proc Natl Acad Sci USA* 77:3932-3936) as a 1.9kb ScaI-NheI fragment. The 1.9kb ScaI-NheI fragment contains the entire coding region for the p15E transmembrane protein and the majority of the coding region for the gp70 SU protein. The 5'-protruding ends are digested with S1-nuclease, and HindIII linkers (5'-CCAAGCTTGG-3'; SEQ ID NO: 2) are added. The env gene is cloned as a HindIII fragment in the HindIII site of pLNCX\* to form plasmid LNCenvpA. The orientation of the HindIII env fragment is such that it can be transcribed and expressed from the cytomegalovirus (CMV) promoter.

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Modification of LNCenvpA to LNCenv

LNCenvpA is cloned as an XbaI fragment in phagemid pBluescript II SK+ for additional site directed mutagenesis (Figure 3). The env encoding HindIII fragment contains a polyadenylation signal that may interfere with the polyadenylation signal provided by the viral vector. The AAUAAA polyadenylation signal is therefore changed to AAGAAA by site directed mutagenesis with the oligonucleotide 5'-GTTTTCTTTTATC-3' (SEQ ID NO: 3). The HindIII site located at the 3' end of the env gene is eliminated by site directed mutagenesis with the oligonucleotide 5-CAAGCATGGCTTGCC-3' (SEQ ID NO: 4). The env containing retroviral vector is recircularized by XbaI restriction and ligation to form plasmid LNCenv.

15 Molecular cloning of anti-CEA immunoglobulin genes

cDNA encoding the mature variable region domain of anti-CEA heavy chain genes is cloned as an XhoI-SpeI fragment using the polymerase chain reaction (PCR) and RNA template. RNA is derived from the spleen of mice immunized against purified carcinoembryonic antigen. Alternatively, RNA can be derived from hybridoma cell lines that secrete monoclonal antibodies against CEA, e.g., 1116NS-3d (American Type Culture Collection CRL8019) or CEA 66-E3 (Wagener, C., et al., 1983, *J Immunol* 130:2308-2315).

The following PCR primers hybridize to cDNA encoding the aminoterminal end of mature heavy chain genes (Stratocyte, Inc.). The degenerate primers introduce an XhoI site which is underlined.



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5' AGGTGCAGCTGCTCGAGTCGGG 3' (SEQ ID NO: 5)

5' AGGTGCAACTGCTCGAGTCGGG 3' (SEQ ID NO: 6)

5' AGGTGCAGCTGCTCGAGTCTGG 3' (SEQ ID NO: 7)

5' AGGTGCAACTGCTCGAGTCTGG 3' (SEQ ID NO: 8)

5' AGGTCCAGCTGCTCGAGTCTGG 3' (SEQ ID NO: 9)

XhoI

The following PCR primer hybridizes to immunoglobulin heavy chain mRNA within the region coding for the J-region and introduces SpeI and BstEII sites.

10 5' CTATTAACTAGTGACGGTTACCGTGGTCCCTTGGCCCCA 3' (SEQ ID NO: 10)

SpeI BstEII

The amplified anti-CEA variable heavy chain DNA is cloned as an XhoI-SpeI fragment in an ImmunoZAP H vector (Stratagene, Inc.) (Mullinax, R.I. et al., 1990, *Proc Natl Acad Sci USA* 87:8095-8099). ImmunoZAP H is a modified lambdaZAP vector that has been modified to express in *E.coli* immunoglobulin variable heavy chain fragments behind a pelB signal sequence. The procedure could similarly be performed by expressing immunoglobulin variable light chain fragments in a packaging cell line.

#### Identification of high affinity anti-CEA clones

Clones expressing high affinity anti-CEA antibodies are identified by a filter binding assay. The anti-CEA phage library is screened by nitrocellulose plaque lifts with [<sup>125</sup>I]bovine serum albumin conjugated to CEA, as previously described (Huse, W.D., et al., 1989, *Science* 246:1275-1281). High and intermediate affinity anti-CEA clones are chosen for further manipulation.

#### Construction of plasmid LNC-immunoglobulin

Two strategies are presented for creating plasmid LNC-immunoglobulin (in this example, LNC-antiCEA, which codes for an anti-CEA immunoglobulin gene). LNC-immunoglobulin vectors encode an immunoglobulin peptide

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fused to an amino-terminal signal sequence. Some amino acids at the amino-terminal end of the mature immunoglobulin peptide have been modified by the PCR primers used to generate the immunoZAP library.

- 5 Moreover, the design of the LNC-antiCEA plasmid results in the insertion of an extra amino acid at the amino terminal end. These amino acid changes do not affect antigen binding because 1) the amino acid changes are conservative; 2) the affected amino acids are normally  
10 variable at those sites; and 3) the affected amino acids occur within the framework region of immunoglobulins which has been shown not to participate in antigen binding or conformation of the antibody (Relchman et al. 1988 *Nature* 332:323-327). It is for these same reasons  
15 that cleavage of the signal sequence from mature peptide will not be affected.

Both strategies for creating LNC-immunoglobulin rely on the use of plasmid pUC Star-Sig, the construction of which is presented below.

- 20 An immunoglobulin signal sequence is cloned into a modified pUC119 vector to create pUC Star-Sig as follows (Figure 4). pUC119 is a phagemid containing a polylinker cloning site. The multiple cloning sites of pUC119 are replaced with new restriction sites by insertion of the  
25 following polylinker into the HindIII and XbaI sites of pUC119.

HindIII PstI XhoI BclI SpeI NotI ClaI XbaI  
5'-AGCTTCTGCAGGCTCGAGTGATCAACTAGTGCGGCCGCATCGATT-3' (SEQ  
ID NO: 11)  
30 3'-AGACGTCCGAGCTCACTAGTTGATCACGCCGGCGTAGCTAAGATC-5' (SEQ  
ID NO:12)

The modified pUC119 is called pUC Star-1 (Figure 4). The restriction sites may be further separated by small

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linkers, if adjacent restriction sites interfere with one another during digestion.

The signal sequence from an anti-NP immunoglobulin heavy chain gene is isolated from plasmid pcDFL.1 (Ucker, D.S., et al., 1985, *J Immunol* 135:4204-4214) as a ~330bp PstI fragment. The 330bp PstI fragment is subcloned into pUC Star-1 to yield plasmid pUC Star-Sig (Figure 4). The PstI fragment is oriented so that the signal sequence can be expressed.

10     A. Construction of LNC-immunoglobulin through  
       plasmid LNC-Sig, an immunoglobulin expression vector.

LNCX\* is converted into a eukaryotic immunoglobulin expression vector (Figures 4 and 5). An immunoglobulin heavy chain signal sequence and XhoI-SpeI cloning sites are inserted behind the CMV promoter of  
15     plasmid LNCX\* to allow expression of the PCR amplified immunoglobulin genes. Conversion of LNCX\* is as follows.

The immunoglobulin heavy chain signal sequence is recovered from pUC Star-Sig as a HindIII-ClaI restriction  
20     fragment and cloned into the HindIII-ClaI sites of LNCX\*. The resulting plasmid, LNC-Sig contains a retroviral vector with the immunoglobulin heavy chain signal sequence under control of the CMV promoter (Figure 5).

An anti-CEA gene from the immunoZAP library is  
25     then subcloned into LNC-Sig to form plasmid LNCanti-CEA. This generates an anti-CEA variable heavy chain gene containing a signal sequence (Figure 6). The anti-CEA gene is first excised from immunoZAP phage DNA as a Bluescript SK- phagemid (see lambdaZAP protocols,  
30     Stratagene, Inc. La Jolla, CA). The anti-CEA gene is purified as an XhoI-SpeI fragment and ligated to XhoI-SpeI restricted LNC-Sig. LNC-Sig contains three SpeI sites. Therefore, to generate plasmid LNCanti-CEA, the ligation mix is transformed into neomycin-sensitive,  
35     ampicillin-sensitive E. coli and neomycin-resistant,

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ampicillin-resistant transformants are selected for. Plasmid LNCanti-CEA is screened from neomycin-resistant, ampicillin-resistant transformants by using the SpeI-XhoI anti-CEA restriction fragment from Bluescript SK-anti-CEA  
5 as a probe. The SpeI site is used because of dependence upon the available sites in the ImmunoZap expression vector. To simplify construction of LNCanti-CEA, a unique NotI site can be introduced into the ImmunoZap H expression vector so that NotI sites can be used instead  
10 of SpeI sites.

B. Construction of LNC-immunoglobulin through plasmid pUC Star-Sig

An anti-CEA gene from the immunoZAP library is subcloned into plasmid pUC Star-Sig to form plasmid pUC  
15 Star-anti-CEA. This generates an anti-CEA variable heavy chain gene containing a signal sequence (Figure 12). The anti-CEA gene is excised from immunoZAP phage DNA as a Bluescript SK-phagemid (see lambdaZAP protocols, Stratagene, Inc., La Jolla, CA). Bluescript SK-anti-CEA  
20 double stranded DNA is prepared and restricted with XhoI and SpeI. The anti-CEA containing XhoI-SpeI fragment is purified by electroelution and ligated to Xho-SpeI restricted pUC Star-Sig to create plasmid pUC Star-antiCEA (Figure 12).

25 The antiCEA gene is transferred from pUC Star-antiCEA to LNCX\* as a HindIII-ClaI fragment to create plasmid LNC-antiCEA (figure 13). The antiCEA-containing HindIII-ClaI fragment is purified from pUC Star-antiCEA by electroelution. Phosphatase treated, HindIII-ClaI  
30 restricted LNCX\* is ligated with the purified HindIII-ClaI antiCEA fragment to generate LNC-antiCEA (figure 13).

Strategy for generating targeted viruses

The starting materials for generation of targeted  
35 viruses are the LNCenv and LNC-immunoglobulin (in this

example, LNC-antiCEA) plasmids shown in Figure 7. .  
Figures 8-11 diagram the general principle for the  
primary generation of targeted viruses. Hybrid  
immunoglobulin-env proteins are generated that target  
5 viruses to cells expressing carcinoembryonic antigen.  
Since the location of important determinants for envelope  
protein sorting (S), trimerization (T), and fusion (F) is  
not known with certainty, the immunoglobulin gene is  
ligated to progressive deletions of the env gene and  
10 functional immunoglobulin-env hybrids are selected for.

#### Useful Envelope Fragments or Analogs

The envelope portion of the fusion protein may  
consist of any portion of the envelope protein (or any  
analog thereof) which is sufficient to direct efficient  
15 incorporation of the envelope fusion protein into the  
viral coat (upon budding of the recombinant virus from a  
producer cell line). Such fragments or analogs may be  
determined using the following general selection scheme  
which generally involves ligation of cell surface  
20 receptor binding domains of ligand genes to progressive  
deletions of viral envelope genes. The correct  
combination of ligand and envelope sequences is  
determined by a selection scheme for the production of  
biologically active targeted virus. The selection scheme  
25 not only produces targeted virus but simplifies the  
construction of future targeted viruses.

#### Construction of hybrid immunoglobulin-env genes in vitro

Plasmid LNCenv contains the coding region for the  
Mo-MuLV env polyprotein (Figure 8). LNCenv is first  
30 linearized by HindIII restriction. A range of deletions  
extending into the env gene is created by collecting  
aliquots of Exonuclease III treated DNA over time and  
removing 5'-processive ends with S1-nuclease (Guo, I.H.,  
et al., 1983, *Methods Enzymol* 100:60; and Sambrook, J.,  
35 et al., 1989, Molecular Cloning, Cold Spring Harbor

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Laboratory Press, Cold Spring Harbor). NotI linkers (5' AGCGGCCGCT 3' SEQ ID NO: 13) are ligated onto the blunt end termini and restricted with NotI. This results in a NotI restriction overhang at the 5'-border of every deletion within the env gene. The NotI overhangs at the other end of the molecules are removed by SalI restriction of the reaction mixture. The reaction mixture is then treated with phosphatase to prevent circularization of the reaction products.

10 The reaction mixture is ligated to a SalI-NotI restriction fragment from LNC-antiCEA that contains the anti-CEA variable heavy chain gene. This creates a pool of functional retroviral vectors encoding an anti-CEA peptide fused to a series of env deletions.

15 Generation of pooled virus constructions

The total reaction mixture from above is transformed into ampicillin-sensitive E. coli and ampicillin resistance is selected for (Figure 9). Recombinants containing functional retroviral vectors are selected for since only they contain the ampicillin resistance gene. Plasmid DNA is prepared from transformants grown in liquid culture to create a pool of retroviral vectors containing different immunoglobulin-env fusion genes.

25 The DNA is transfected into the crip2 retroviral packaging cell line (Danos, O., et al., 1988, *Proc Natl Acad Sci USA* 85: 6460-6464). Alternatively, DNA is transfected into a packaging cell line that does not encode wild-type env protein. The transfected packaging cell line synthesizes each of the different hybrid immunoglobulin-env proteins as well as the wild type env protein (encoded by an env gene contained in the cell line). The transfected packaging cell line secretes a pool of enveloped retroviruses containing the different retroviral genomes encoding hybrid immunoglobulin-env

30  
35

genes. If the hybrid immunoglobulin-env protein retained all of the necessary determinants for efficient incorporation into viral envelopes then the hybrid-env protein can be incorporated into viral envelopes. Wild type env proteins encoded for by the packaging cell line are also incorporated into the viral envelopes. This creates a virus containing both wild type and hybrid env proteins in the viral envelope. This system therefore selects for immunoglobulin-env hybrids that can incorporate their gene products into the viral envelope.

Virus pools are harvested from media filtered at 0.45 $\mu$  to remove contaminating G418-resistant packaging cells.

#### Selection and characterization of targeted virus

G418-sensitive target cells are exposed to virus pools by standard procedures, and G418-resistant cells are selected for. The target cells can be any non-mouse cell line (uninfectable by wild type Mo-MuLV) that expresses carcinoembryonic antigen. Examples include ATCC COLO 205, a human cell line isolated from the ascites of a patient with carcinoma of the colon (A.T.C.C.#CCL 222); LR-73 CEA, a chinese hamster ovary cell line transfected with a mouse carcinoembryonic antigen gene (Benchimol, S. et al., *supra*); and HCT48, a human colon adenocarcinoma cell line (Shi, Z.R., et al., 1983, *Cancer Res* 43:4045-4049).

G418-resistant cells can only have arisen from transduction of the neomycin resistance gene by targeted virus. This system therefore selects for recombinant viruses that have hybrid immunoglobulin-env proteins that have retained all the necessary determinants for viral targeting and fusion.

#### Rescue of integrated immunoglobulin-env gene

Infection by targeted virus results in integration of the hybrid envelope gene that created the targeting

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protein. The integrated hybrid immunoglobulin-env gene is rescued from the host DNA by polymerase chain reaction (PCR) with the following primers:

PCR 5' Rescue primer:

5 5'-CCAGCCTCCGCGGCCCAAGCTTCTGCA-3' (SEQ ID NO: 14)  
HindIII

PCR 3' Rescue primer:

5'-GGTTCTCTAGAACTGCTGAGGGC-3' (SEQ ID NO: 15)  
XbaI

10 PCR amplification with these primers generates the  
immunoglobulin-env gene bordered by HindIII and XbaI  
sites. The amplified DNA is restricted with HindIII and  
XbaI to create sticky ends and the DNA is ligated into  
HindIII-XbaI cut LNCX\*. When transfected into crip2  
15 packaging cells, this generates a retroviral vector  
targeted to cells expressing cell surface  
carcinoembryonic antigen (e.g., colon cancer cells).

The retroviral vector produced in the above  
selection scheme is targeted to both CEA-expressing human  
20 cells (directed by the hybrid envelope protein) and  
normal mouse cells (directed by the wild type envelope  
protein) when produced in crip2 packaging cells. To  
create viruses that infect target cells only, the  
retroviral vector will first be tested to determine if  
25 incorporation of the hybrid envelope protein alone is  
sufficient to direct virus fusion. This is accomplished  
by transfecting DNA into a modified packaging cell line  
that does not encode wild type env.

If fusion functions are found to have been  
30 supplied from the wild type envelope protein, targeted  
viruses will be created as follows. A packaging cell  
line will be created that encodes an env gene containing  
mutations in the receptor binding domain. When



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transfected with the targeted viral vector DNA, targeted viruses expressing both hybrid ligand-env proteins and env proteins with mutated binding sites will be produced. The viruses will exclusively infect target cells.

5 The targeted viral vector is a universal vector

The viral vector that is constructed by the above procedure is a universal targeted vector (Figure 11). Targeting to other cells is accomplished by replacing the XhoI-SpeI anti-CEA fragment with any XhoI-SpeI fragment  
10 encoding an in-frame immunoglobulin or ligand directed against specific cell surface proteins. For example, an XhoI-SpeI immunoglobulin-containing fragment from an immunoZAP library can be fused in frame behind a signal sequence and subcloned into LNCX\* through the pUC Star-  
15 Sig plasmid, as outlined above. Substituting a SalI-NotI fragment from another LNC-immunoglobulin plasmid into the universal vector would create another targeted virus vector.

Other Viral Vectors

20 Any enveloped virus may be used as a vector for the targeted delivery of a therapeutic gene. Particular examples include both DNA and RNA viruses, such as Herpesviridae, e.g., herpes simplex type 1 or 2, Paramyxoviridae, Retroviridae, Hepadnaviridae,  
25 Poxviridae, Iridoviridae, Togaviridae, Flaviviridae, Coronaviridae, Rhabdoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, or Arenaviridae, or any other, yet unclassified, enveloped virus.

An extensive selection of these viruses is  
30 available, e.g., from the American Type Culture Collection.

Targeting Ligands

Any molecule that is capable of directing specific  
interaction with a target host cell (e.g., by specific  
35 recognition of and binding to a host cell surface

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protein) may be used as the targeting ligand portion of the envelope fusion protein. Preferably, such a protein is derived from one member of a ligand:receptor pair. The targeting ligands are not limited to proteins.

5 Carbohydrate and lipid moieties can be attached to the envelope protein via protein fragments containing consensus sequences for glycosylation and lipidation.

Immunoglobulin genes can be used as ligands, as shown in the example above. Genes for high affinity  
10 immunoglobulins are screened from a lambda or bacterial expression library by a filter binding assay with [<sup>125</sup>I] bovine serum albumin conjugated to antigen, as previously described (Huse, et al. *Supra*).

Cell surface molecules such as integrins, adhesion  
15 molecules or homing receptors can be used as cell-specific ligands since they are involved in cell-cell interactions via receptors on other cells. Genes encoding these molecules can be identified by the panning method of Seed and Aruffo (Seed. B., et al., 1987, *Proc*  
20 *Natl Acad Sci USA* 84:3365-3369).

Hormones that bind to specific receptors can be used as targeting ligands as well as viral proteins, such as HIV envelope protein gp120, and modifications of naturally occurring ligands.

#### 25 Therapeutic Genes

Therapeutic genes useful in the invention include the following. 1) Genes that are therapeutic to cancer cells may include a) antisense oncogenes; b) tumor suppressor genes, such as p53 or the retinoblastoma gene  
30 product Rb, c) destructive toxin genes such as a diphtheria toxin gene; d) cytokines such as tumor necrosis factor or interferons; or e) any other therapeutic gene. 2) Therapeutic genes targeted to cells that are infected with HIV. Specific examples  
35 include antisense DNA complementary to essential genes

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for HIV, e.g., polymerase; destructive toxin genes; e.g., diphtheria toxin; and genes that will invoke intracellular immunity, e.g., HIV enhancer sequences that titrate and remove HIV regulatory proteins (Baltimore, D., 1988, *Nature* 335:395-396). 3) Genes to correct inherited deficiencies. Examples include, but are not limited to, insulin genes delivered specifically to pancreatic beta cells, or the cystic fibrosis transmembrane regulator (CFTR) gene delivered to the appropriate lung cells of cystic fibrosis patients. The expression of targeted genes can be further accomplished through the use of tissue specific enhancers that regulate the transgene.

#### Therapy

For any gene therapy described herein, the appropriate recombinant virus, as described above, is administered to a patient in a pharmaceutically-acceptable buffer (e.g., physiological saline). The therapeutic preparation is administered in accordance with the condition to be treated. For example, to treat an HIV-infected individual, the virus is administered by direct injection, e.g., by intravenous, intramuscular, or intraperitoneal injection, at a dosage that provides suitable targeting and lysis of HIV-infected host cells. Alternatively, it may be necessary to administer the targeted virus surgically to the appropriate target tissue, or via a catheter, or a videoscope. It may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or spray. Again, an appropriate dosage is an amount of therapeutic virus which effects a reduction in the disease.

Targeted virus can also be administered by implanting viral packaging cells into a patient. The cells can be enclosed in a semi-permeable container, e.g., permeable to a virus but not permeable to a

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packaging cell. The implanted container may be removable. Alternatively, the container may be hooked up to a patient intravenously, so that virus enters the patient through a needle or through a catheter. In this way the patient receives a continuous dose of viral gene therapy.

#### Other Embodiments

Other embodiments are within the following claims.

For example, replication competent viruses may be used in certain cases. In other cases, where replication-deficient viruses are necessary, it may be efficacious to administer modified packaging cells, rather than the targeted virus, to patients. By this method a non-proliferating dose of recombinant virus is delivered to a local area, and then the virus locates the specific target cell. For example, tumor infiltrating lymphocytes (TIL), which surround cancer cells, can be modified to secrete locally high concentrations of cancer cell-targeted virus. Treatment may be repeated as necessary. Immune response against targeted viruses can be overcome with immunosuppressive drugs.

In addition to colon cancer cells, the virus of the invention may be used to target other cancer cells, e.g., ovarian, breast, or lung cancer cells, or cells affected with hereditary diseases such as muscular dystrophy, Huntington's disease, or cells with a defect in adenosine deaminase. Herpesviridae viruses may include Herpes simplex type 1 or type 2, Epstein-Barr virus, or Cytomegalovirus. Sendai virus and Vaccinia virus may also be adapted to this method.

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SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Alexander T. Young  
(ii) TITLE OF INVENTION: GENE THERAPY USING  
TARGETED VIRAL VECTORS  
(iii) NUMBER OF SEQUENCES: 15  
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(F) ZIP: 02110-2804

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb  
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX  
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.00)  
(D) SOFTWARE: WordPerfect (Version 5.1)

## (vi) CURRENT APPLICATION DATA:

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- 36 -

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCAGAAGGTC GACCCAACG

19

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCAAGCTTGG

10

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTTTTCTTTT ATC

13

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CAAGCATGGC TTGCC

15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGTGCAGCT GCTCGAGTCG GG

22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGTGCAACT GCTCGAGTCT GG

22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGGTGCAGCT GCTCGAGTCT GG

22

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## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGGTGCAACT GCTCGAGTCT GG 22

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGGTCCAGCT GCTCGAGTCT GG 22

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTATTAACTA GTGACGGTTA CCGTGGTCCC TTGGCCCCA 39

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGCTTCTGCA GGCTCGAGTG ATCAACTAGT GCGGCCGCAT CGATT 45

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	45
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGACGTCCGA GCTCACTAGT TGATCAGCC GCGGTAGCTA AGATC 45

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	10
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGCGGCCGCT 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCAGCCTCCG CGGCCCAAG CTTCTGCA 28

- 40 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	24
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGTTCTCTA GAAACTGCTG AGGGC

24

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What is claimed is:

Claims

1. A method for expressing a nucleic acid  
of interest in a heterologous host cell, said method  
5 comprising
  - (a) providing a virus whose genome comprises  
(i) said nucleic acid of interest and (ii) a hybrid  
envelope gene, said hybrid gene encoding an envelope  
fragment joined to a targeting ligand, whereby said  
10 envelope fragment does not facilitate recognition or  
binding of its normal host cell but which does facilitate  
efficient incorporation of said virus into a mature viral  
particle and whereby said targeting ligand facilitates  
targeting and binding of said mature viral particle to  
15 the surface of said heterologous host cell, and  
(b) administering said virus so as to permit  
viral infection of said heterologous host cell.
2. The method of claim 1, wherein said  
virus is an envelope virus.
- 20 3. The method of claim 2, wherein said  
envelope virus is a Herpesviridae.
4. The method of claim 2, wherein said  
envelope virus is a Retroviridae.
5. The method of claim 4, wherein said  
25 Retroviridae is a Moloney murine leukemia virus.
6. The method of claim 1, wherein said  
nucleic acid of interest is DNA.
7. The method of claim 1, wherein said  
nucleic acid of interest is RNA.

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8. The method of claim 1, wherein said heterologous host cell is infectious.

9. The method of claim 1, wherein a portion of said hybrid envelope fragment consists of a receptor  
5 binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

10. The method of claim 9, wherein said envelope fragment further consists of a fusion domain.

11. The method of claim 1, wherein the fusion activity of said envelope fragment is performed by  
10 a second protein.

12. The method of claim 1, wherein said administration is by implanting a container enclosing  
15 said virus into a patient.

13. The method of claim 12, wherein said virus is inside a packaging cell.

14. A virus, the genome of which encodes a hybrid envelope protein, said hybrid protein comprising  
20 an envelope fragment joined in frame to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of said hybrid envelope protein into a mature viral particle and  
25 whereby said non-viral protein facilitates targeting and binding of said mature viral particle to the surface of a cell not normally infected by said virus.

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15. The virus of claim 14, wherein said virus is an envelope virus.

16. The virus of claim 15, wherein said envelope virus is a Herpesviridae.

5 17. The virus of claim 15, wherein said envelope virus is a Retroviridae.

18. The virus of claim 17, wherein said Retroviridae is a Moloney murine leukemia virus.

10 19. The virus of claim 14, wherein said nucleic acid of interest is DNA.

20. The virus of claim 14, wherein said nucleic acid of interest is RNA.

21. The virus of claim 14, wherein said heterologous host cell is infectious.

15 22. The virus of claim 14, wherein a portion of said hybrid envelope protein consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

20 23. The virus of claim 22, wherein said envelope fragment further consists of a fusion domain.

24. The virus of claim 14, wherein the fusion activity of said envelope fragment is performed by a second protein.

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25. The virus of claim 14, wherein said administration is by implanting a container enclosing said virus into a patient.

26. The virus of claim 25, wherein said virus is inside a packaging cell.

27. A method for delivering a nucleic acid of interest to a heterologous host cell, said method comprising

- a) providing a virus whose genome comprises
  - 10 (i) said nucleic acid of interest and (ii) a hybrid envelope gene, said hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding to its normal host cell but does facilitate
  - 15 efficient incorporation of said virus into a mature viral particle and whereby said targeting ligand facilitates targeting and binding of said mature viral particle to the surface of said heterologous host cell, and
  - b) administering said virus so as to permit
  - 20 viral infection of said cell.

28. The method of claim 27, wherein said virus is an envelope virus.

29. The method of claim 28, wherein said envelope virus is a Herpesviridae.

25 30. The method of claim 28, wherein said envelope virus is a Retroviridae.

31. The method of claim 30, wherein said Retroviridae is a Moloney murine leukemia virus.

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32. The method of claim 27, wherein said nucleic acid of interest is DNA.

33. The method of claim 27, wherein said nucleic acid of interest is an RNA.

5 34. The method of claim 27, wherein said heterologous host cell is infectious.

35. The method of claim 27, wherein a portion of said hybrid envelope fragment consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

10

36. The method of claim 35, wherein said envelope fragment further consists of a fusion domain.

37. The method of claim 27, wherein said the fusion activity of said envelope fragment is performed by a second protein.

15

38. The method of claim 27, wherein said administration is by implanting a container enclosing said virus into a patient.

20 39. The method of claim 38, wherein said virus is inside a packaging cell.

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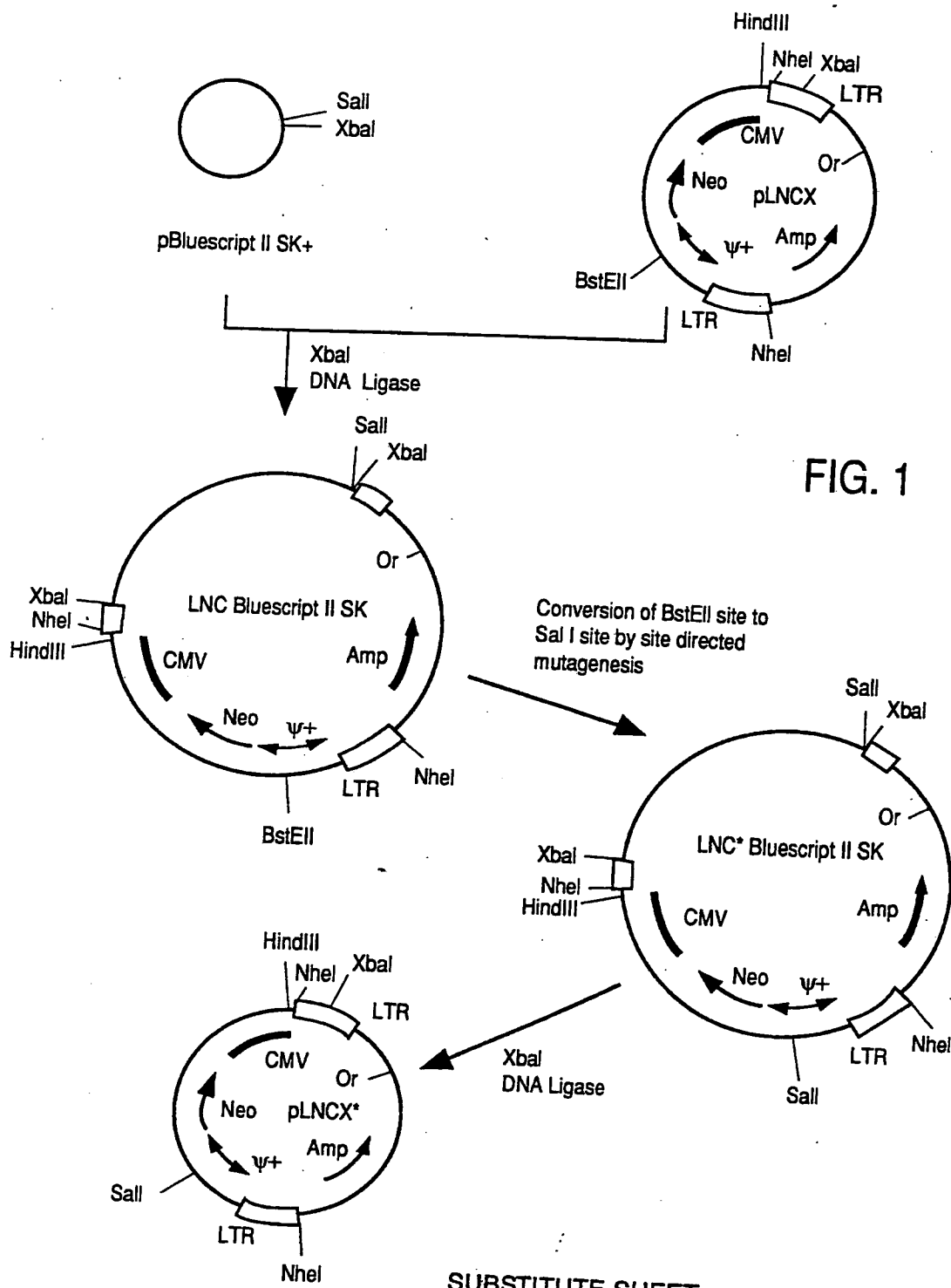


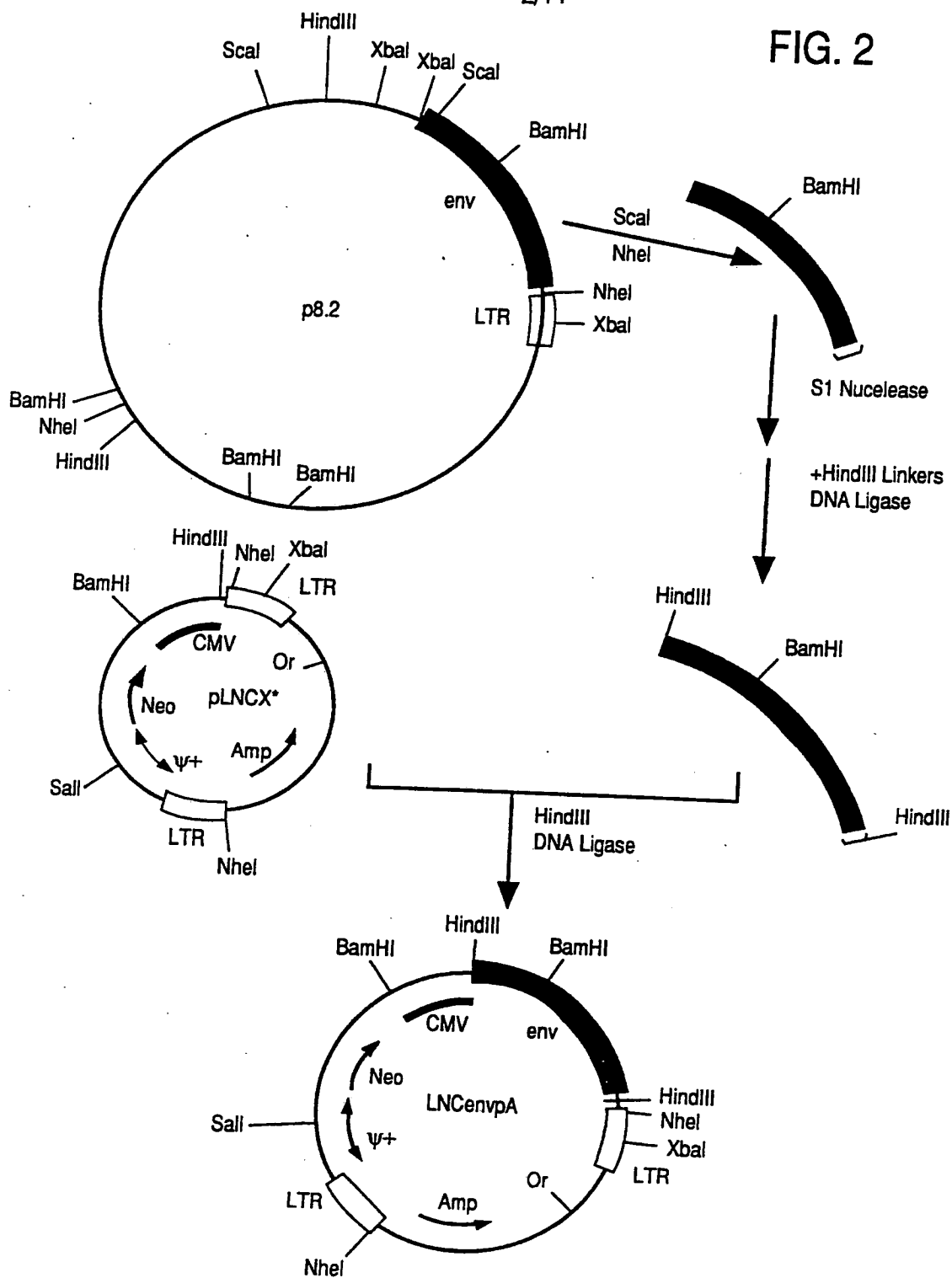
FIG. 1

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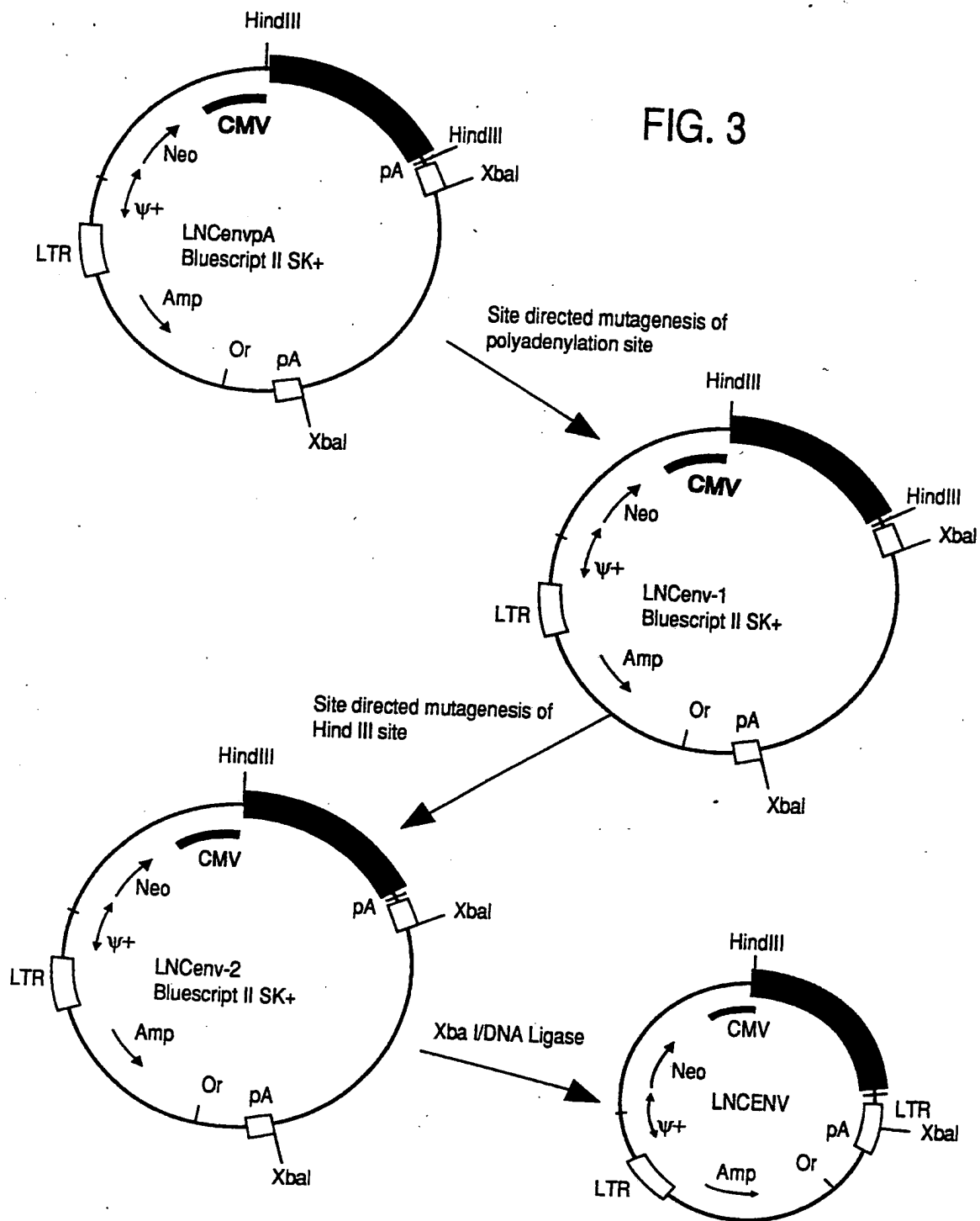
FIG. 2



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FIG. 3



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AccI  
 HincII  
 HindIII SphI PstI SmaI  
 Sall XbaI BamHIXmaI  
 ATGACCATGATTACGCCAAGCTTGCATGCCCTGCAGGTCTGACTCTAGAGGATCC PUC119  
 TACTGGTACTAATGCGGTTCCGAACGTACGGACGTCACGCTGAGATCTCCTAGG  
 KpnI SacI EcoRI  
 CCGGTACCGAGCTCGAATTCACTGGCC  
 GGCCCATGGCTCGAGCTTAAGTGACCCG

HindIII/XbaI  
 Phosphatase  
 +SYNTHETIC POLYLINKER

HindIII PstI XhoI BclI SpeI NotI XbaI  
 AGCTTCTGCAGGCTCGAGTGATCAACTAGTCCGGCCGCGATTCGATT  
 AGACGTCGAGCTCACTAGTTGATCACGCCGGCGTAGCTAAGATC

HindIII PstI XhoI BclI SpeI NotI  
 ATGACCATGATTACGCCAAGCTTCTGCAGGCTCGAGTGATCAACTAGTCCGGCC  
 TACTGGTACTAATGCGGTTCCGAACGTACGGACGTCACGCTCACTAGTTGATCACGCCGG

FIG. 4A

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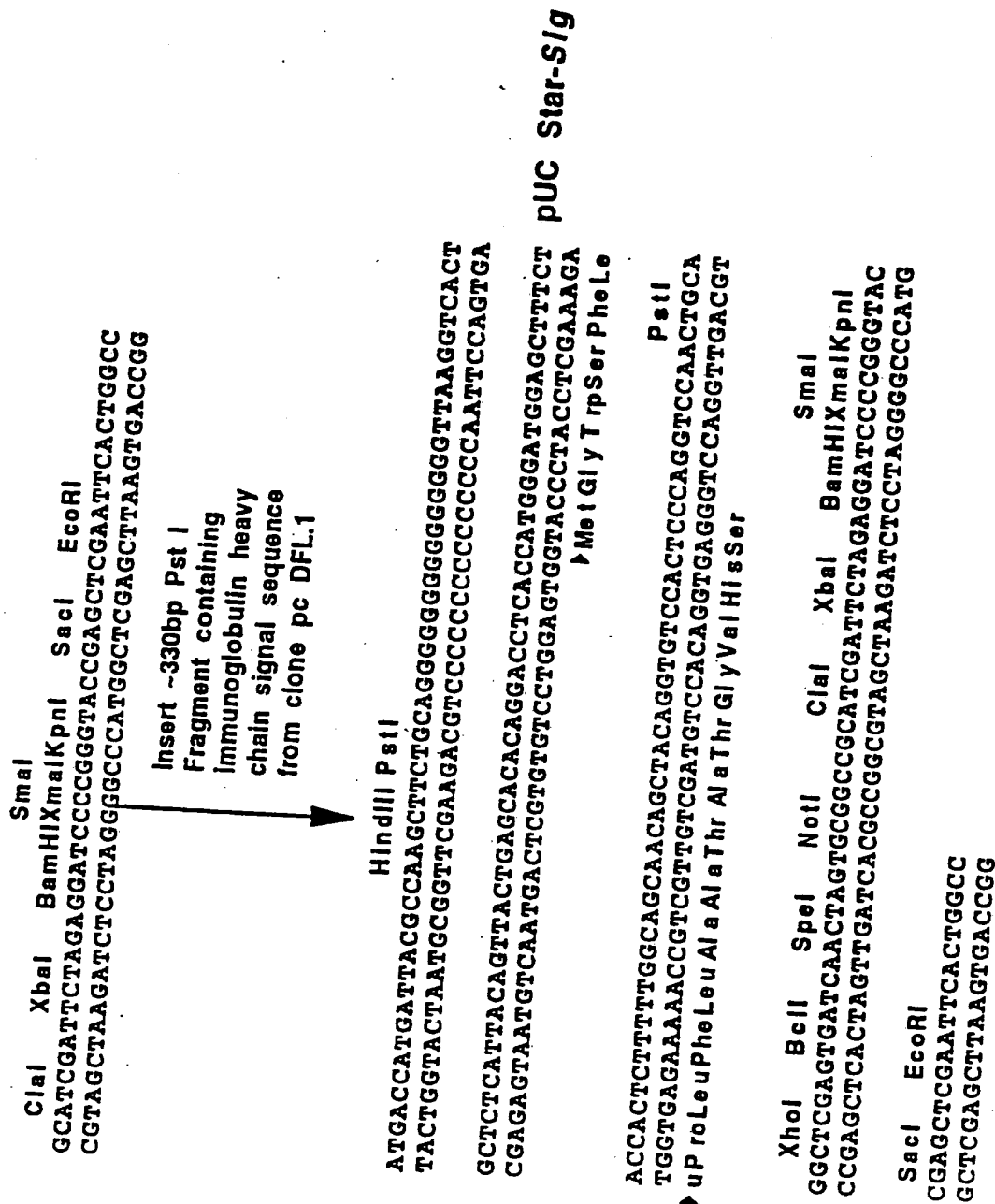


FIG. 4B

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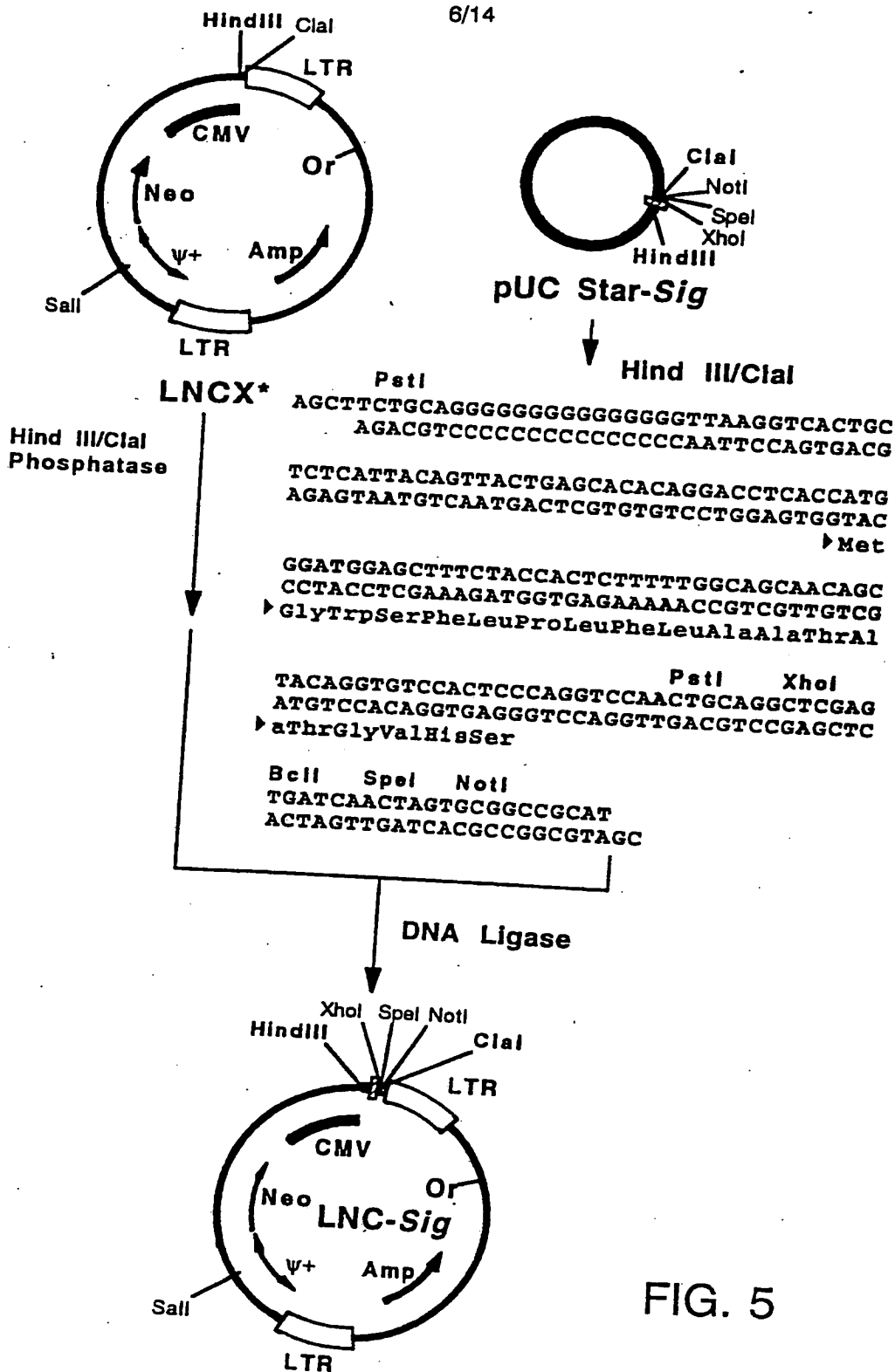
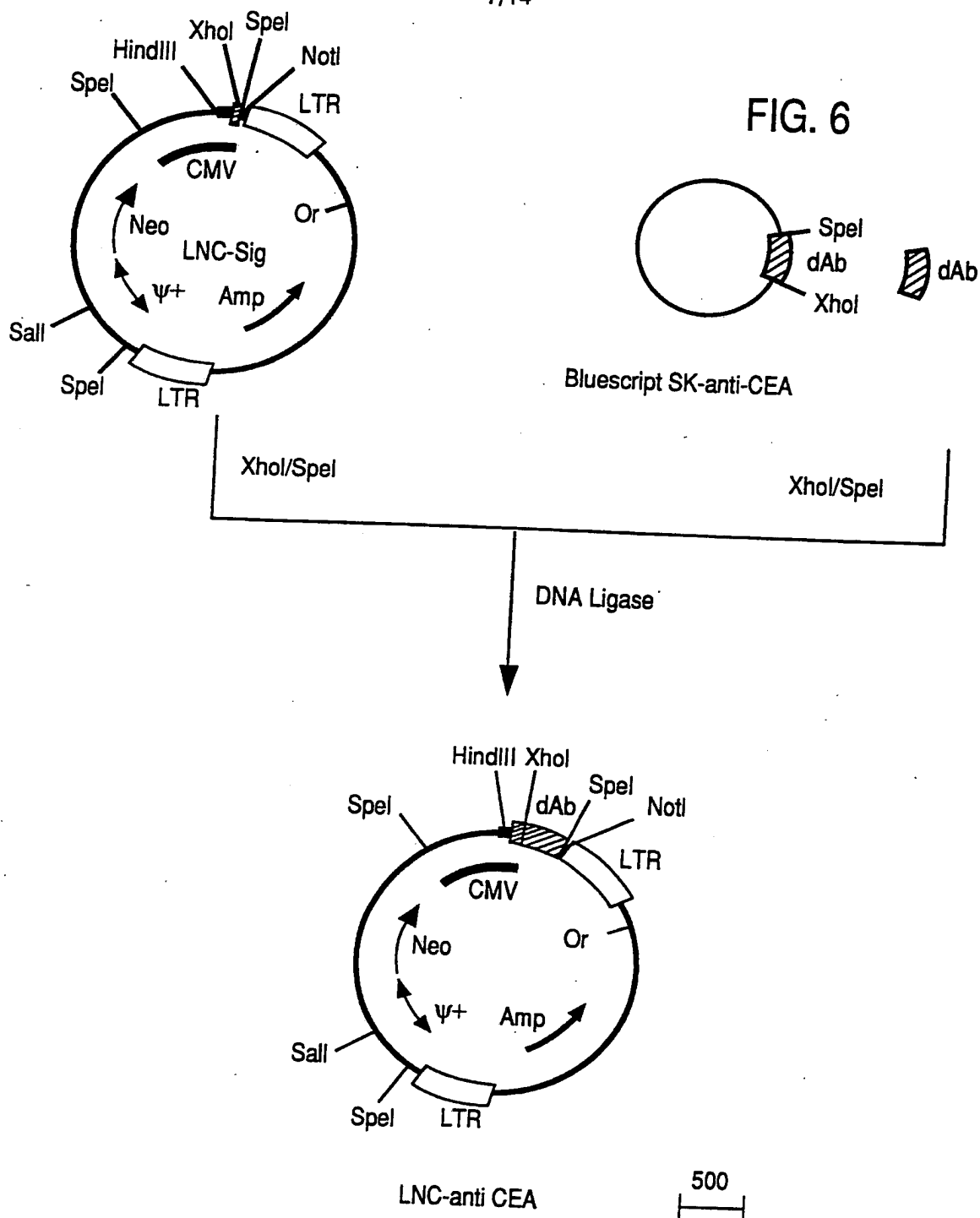


FIG. 5

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FIG. 6



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FIG. 7A

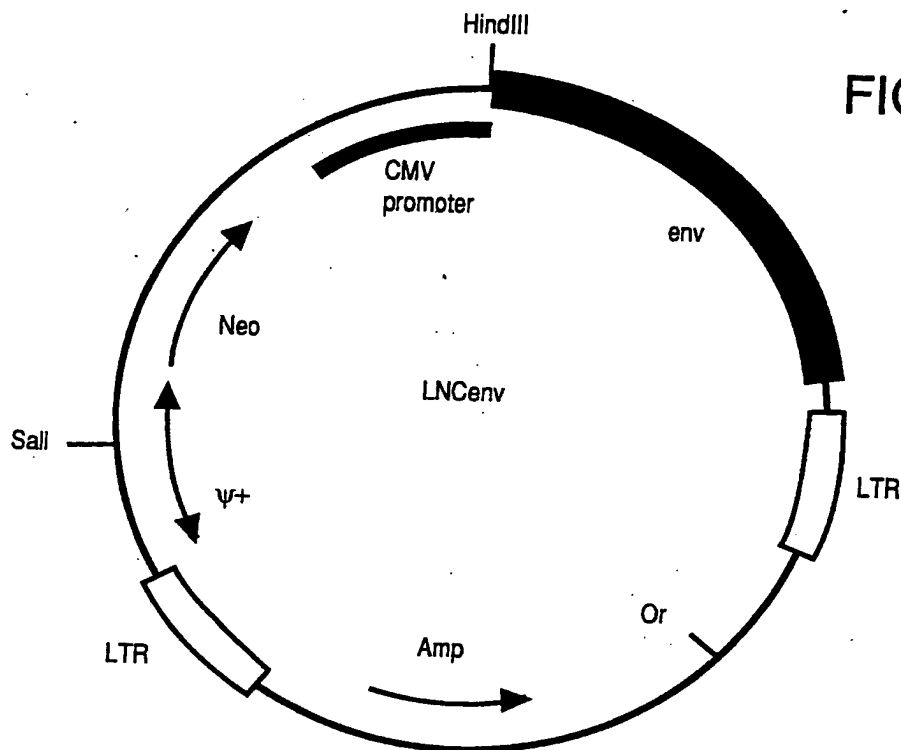


FIG. 7B

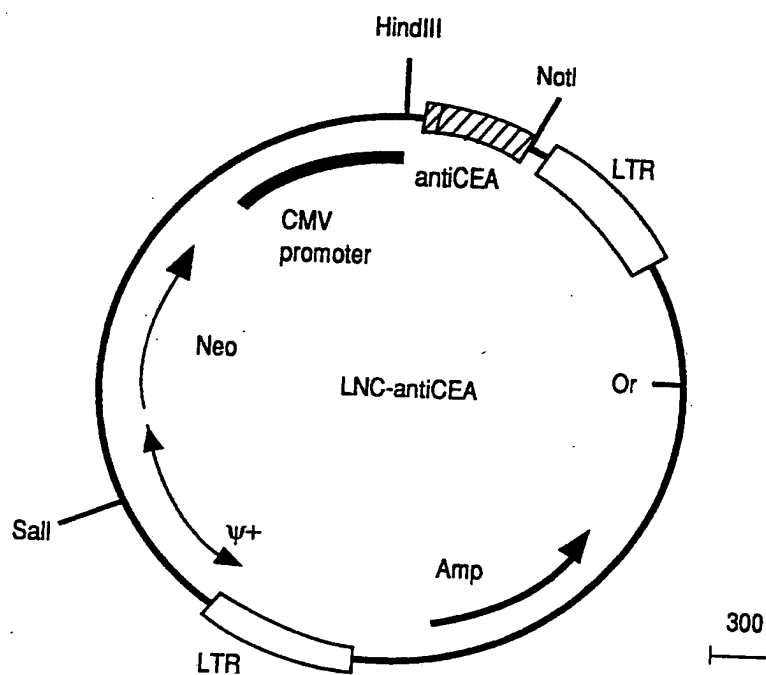
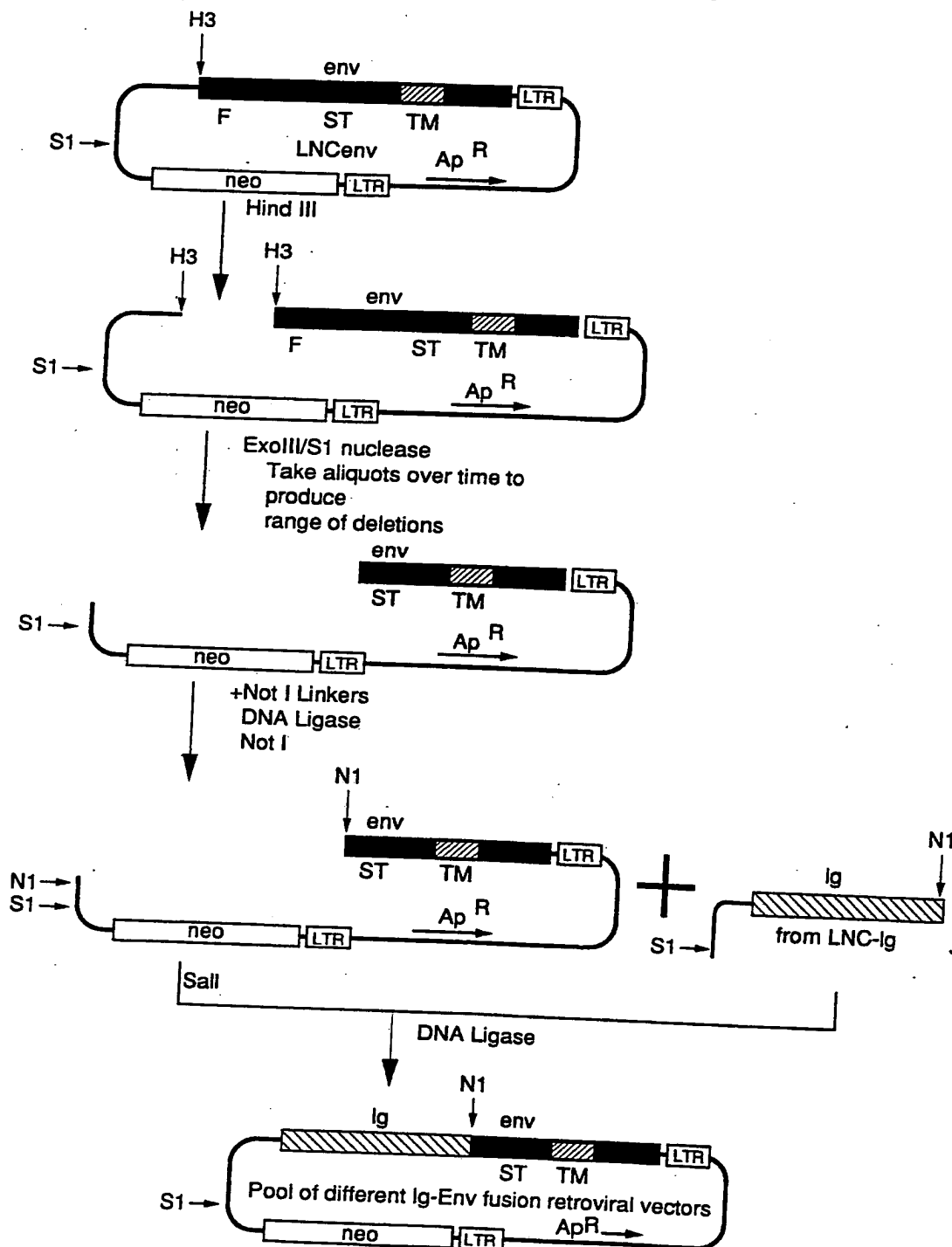


FIG. 8

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**Strategy for generating targeted retroviruses****1. Construction of hybrid Ig-env genes in vitro**

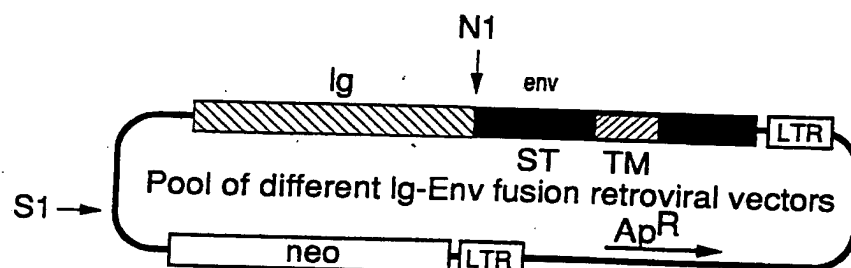
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FIG. 9

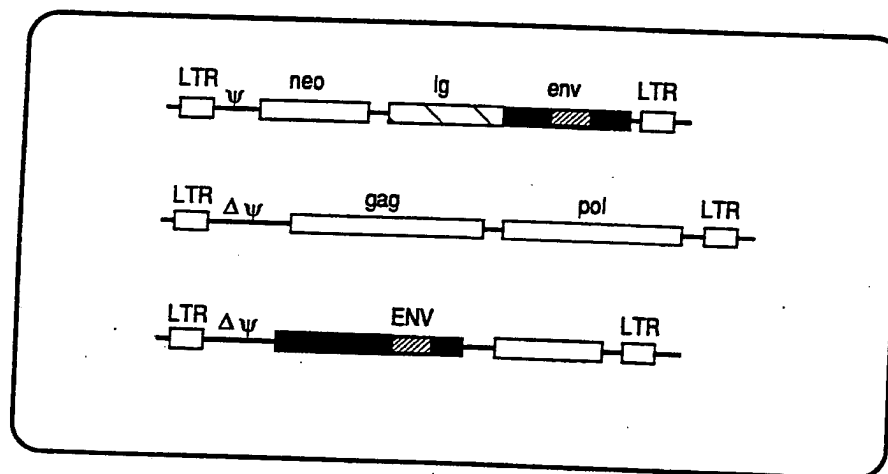
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**Strategy for generating targeted retroviruses**  
II. Generation of pooled virus constructions



Transform ampicillin sensitive E. coli  
Select Ampicillin resistant E. coli in liquid culture  
DNA prep of pool

Transfect crip 2 cells



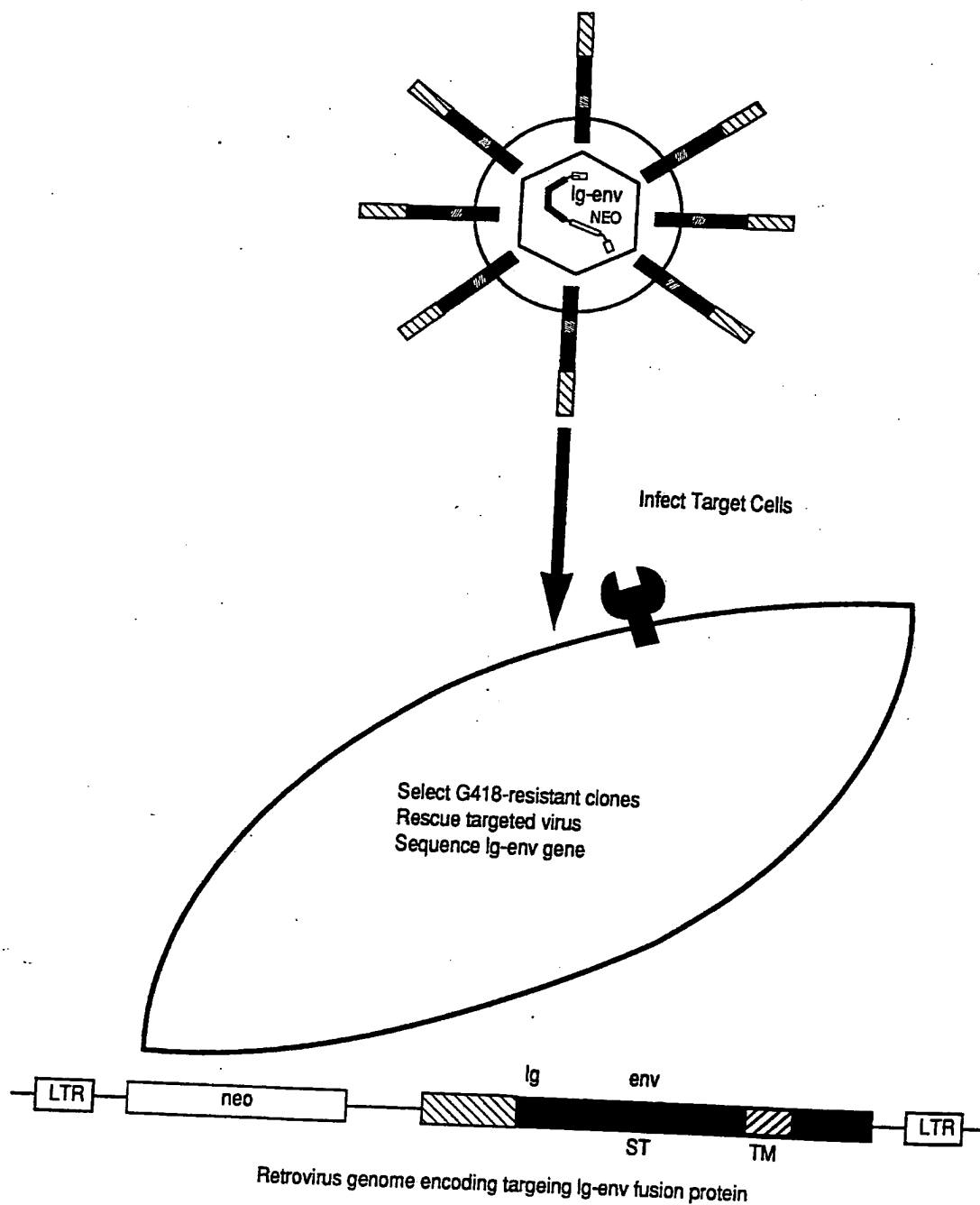
Harvest Virus Pools from  
filtered media

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FIG. 10

**Strategy for generating targeted retroviruses**  
III. Selection and characterization of targeted virus



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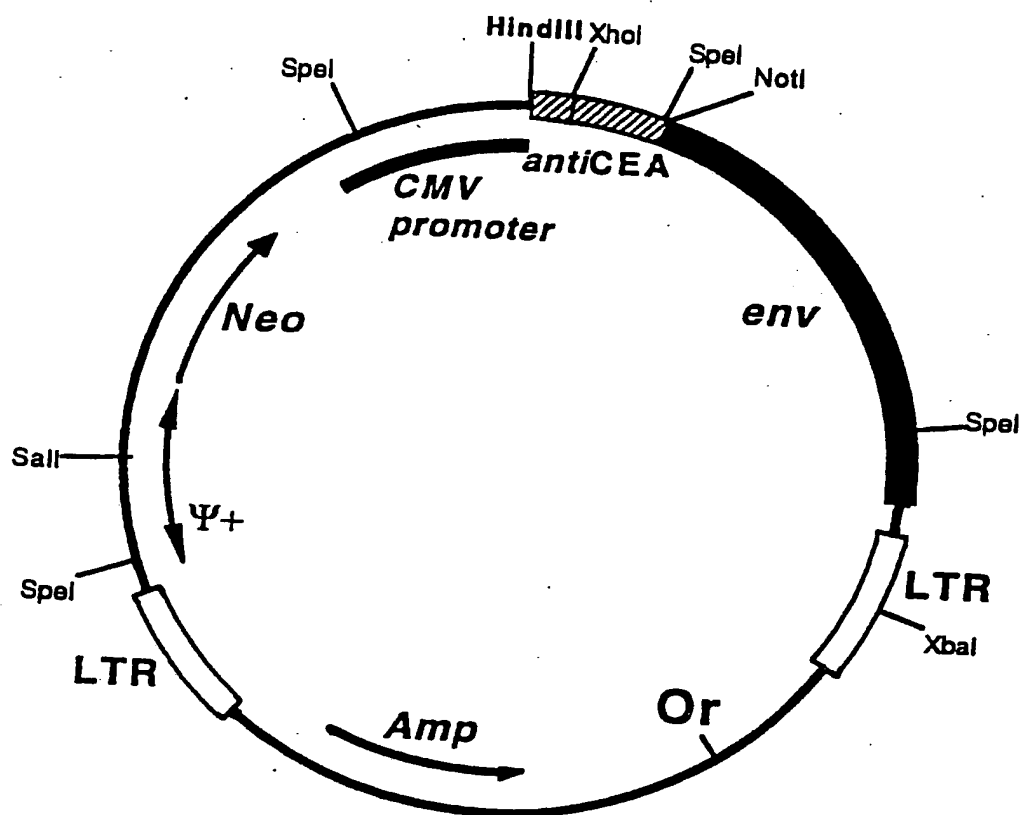
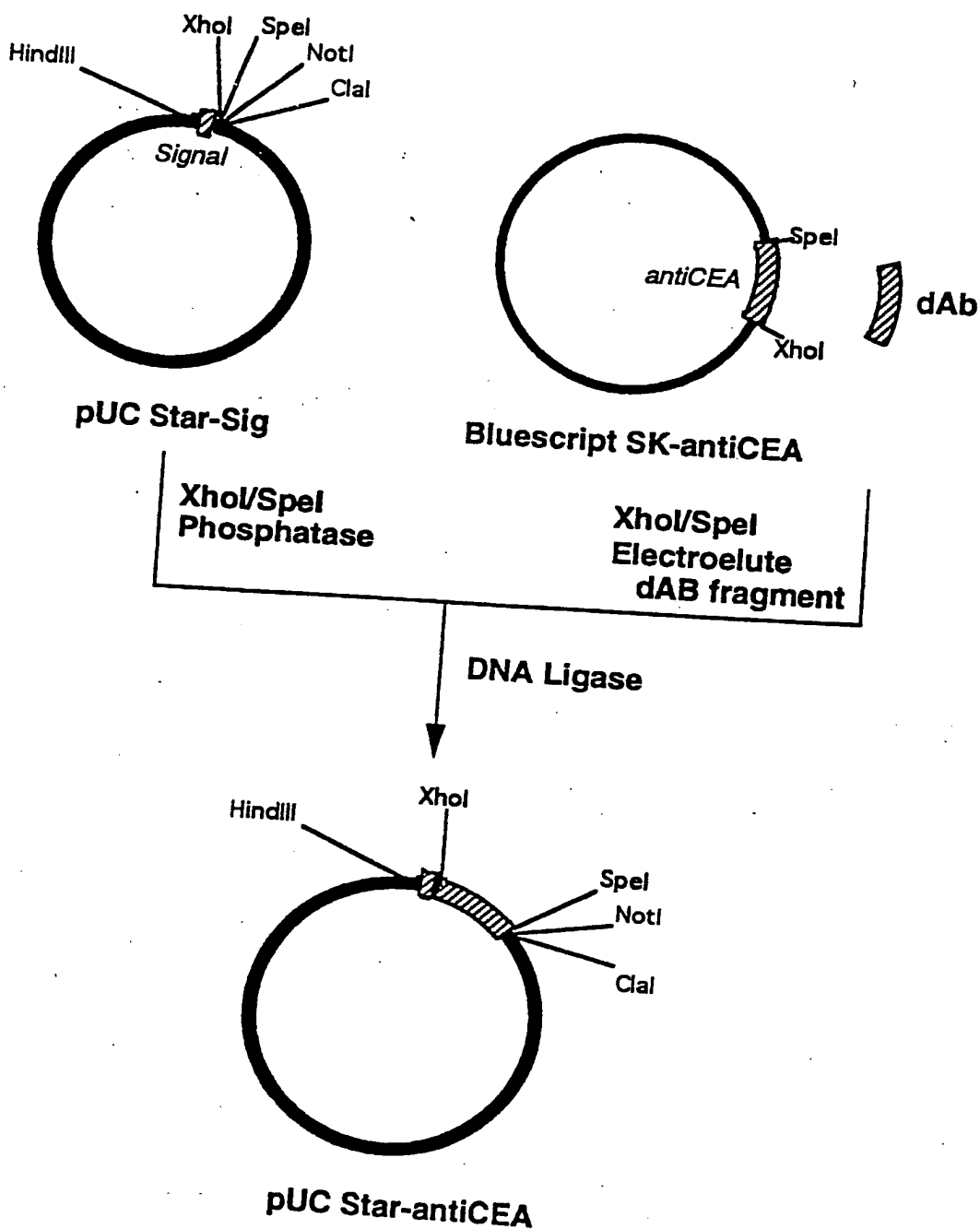


FIG. 11



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FIG. 12  
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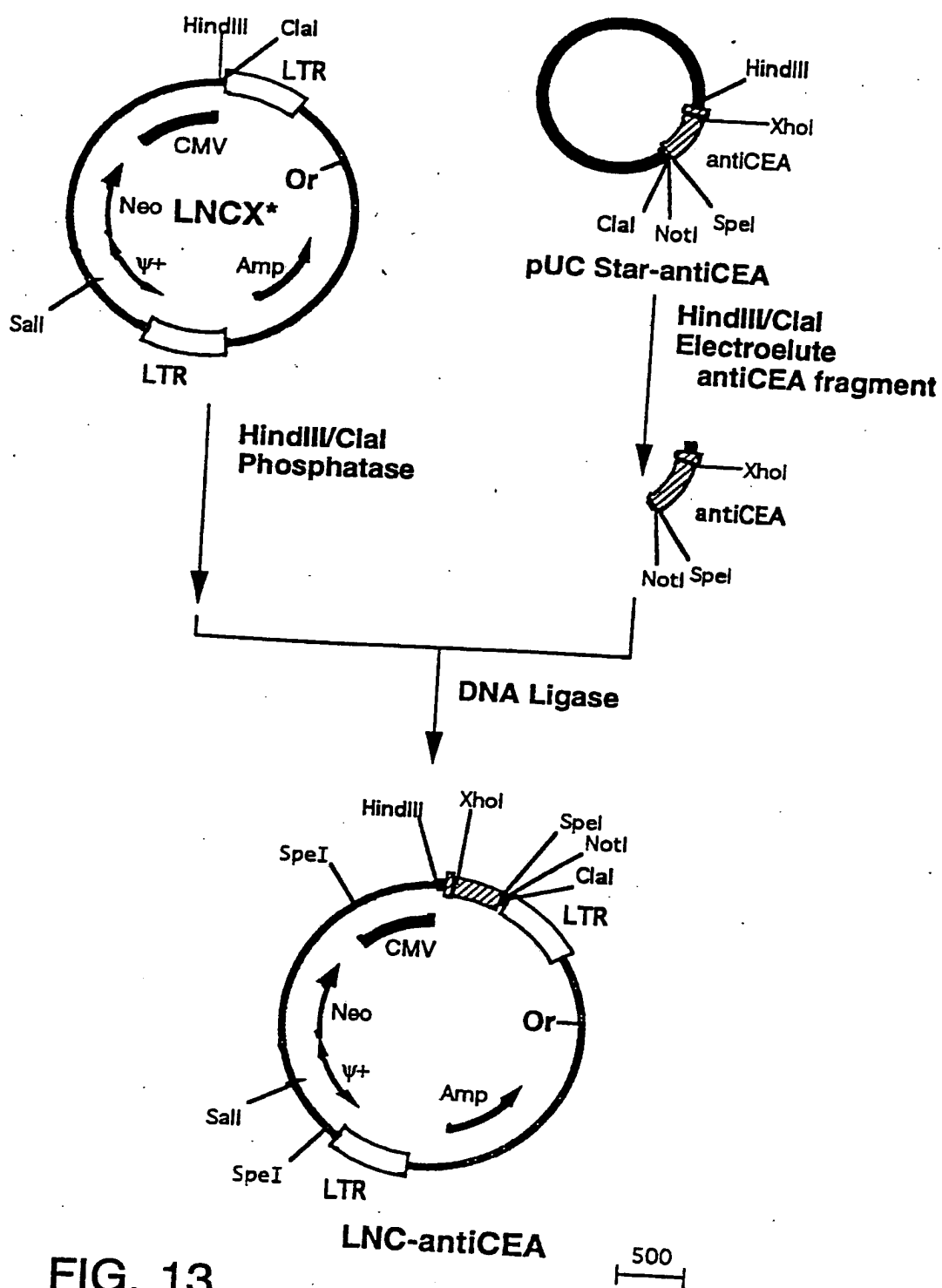


FIG. 13

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02957

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/86; A61K 49/00

US CL : 435/69.1, 320.1; 424/9, 93A

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1; 424/9, 93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
DIALOG DATABASES: CA SEARCH, MEDLINE, BIOSIS PREVIEWS, WORLD PATENTS INDEX; U.S. PATENTS: APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology Today, Vol. 11, No. 6, issued 1990, S. J. Russell, "Lymphokine Gene Therapy for Cancer," pages 196-200. See page 198, second column, first paragraph.	1-39
Y	Science, Vol. 250, issued 07 December 1990, J. A. T. Young et al, "Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles," pages 1421-1423. See entire article.	1-39
Y	Nature, Vol. 340, issued 27 July 1989, J. W. Wills, "Retrosecretion of recombinant proteins," pages 323-324. See page 324, last paragraph.	1-39



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
* "A" document defining the general state of the art which is not considered to be part of particular relevance	* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "E" earlier document published on or after the international filing date	* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* "O" document referring to an oral disclosure, use, exhibition or other means	* "A" document member of the same patent family
* "P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  
15 June 1993Date of mailing of the international search report  
18 JUN 1993Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231Authorized officer  
JOHNNY F. RAILEY II

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02957

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Vol. 244, issued 16 June 1989, T. Friedmann, "Progress Toward Human Gene Therapy," pages 1275-1281. See pages 1277-1278.	1-39

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02957

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
(Form PCT/ISA/206 Previously Mailed.)

- I. Claims 1, 2, 4-11, 14, 15, 17-28, 30-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Retroviridae.
- II. Claims 1-3, 6-11, 14-16, 19-29 and 32-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Herpesviridae.
- III. Claims 12, 13, 38 and 39, drawn to in vivo therapies for expressing nucleic acid or delivery of nucleic acids.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.